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Pro-inflammatory cytokine TNF- α is a key inhibitory factor for lactose synthesis pathway in lactating mammary epithelial cells

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Abstract

Lactose is a milk-specific carbohydrate synthesized by mammary epithelial cells (MECs) in mammary glands during lactation. Lactose synthesis is downregulated under conditions causing inflammation such as mastitis, in which MECs are exposed to high concentrations of inflammatory cytokines. In this study, we investigated whether inflammatory cytokines (TNF- α , IL-1 β , and IL-6) directly influence the lactose synthesis pathway by using two types of murine MEC culture models: the monolayer culture of MECs to induce lactogenesis; and the three-dimensional culture of MECs surrounded by Matrigel to induce reconstitution of the alveolar structure *in vitro*. TNF- α caused severe down-regulation of lactose synthesis-related genes concurrently with the degradation of glucose transporter 1 (GLUT1) from the basolateral membranes in MECs. IL-1 β also caused degradation of GLUT1 along with a decrease in the expression level of β -1,4-galactosyltransferase 3. IL-6 caused both up-regulation and down-regulation of the expression levels of lactose synthesis-related genes in MECs. These results indicate that TNF- α , IL-1 β , and IL-6 have different effects on the lactose synthesis pathway in MECs. Furthermore, TNF- α triggered activation of NF κ B and inactivation of STAT5, suggesting that NF κ B and STAT5 signaling pathways are involved in the multiple adverse effects of TNF- α on the lactose synthesis pathway.

Keywords

TNF- α , IL-1 β , IL-6, lactose, mammary epithelial cell, GLUT1

Abbreviations

α -LA: α -lactalbumin; β 4Galt: β -1,4-galactosyltransferase; DEX: dexamethasone; EGF: epidermal growth factor; GLUT1: glucose transporter 1; HK1: hexokinase 1; LPS: lipopolysaccharide; MEC: mammary epithelial cell; PBST: PBS containing 0.05% Tween 20; PGM: phosphoglucomutase; RT: reverse transcription; STAT5: signal transducer and activator of transcription 5; UGP: UDP-glucose pyrophosphorylase

Introduction

Lactose is a major carbohydrate in milk and provides energy for the healthy growth of infants. Mammary epithelial cells (MECs) in mammary alveoli produce lactose as a milk-specific carbohydrate through the lactose synthesis pathway [1]. MECs take up glucose from the bloodstream as a raw material of lactose via glucose transporter 1 (GLUT1), which is localized in the basolateral membrane of MECs [2]. GLUT12, which is localized in the apical membrane of MECs, also transfers glucose between MECs and milk in the alveolar lumen [3]. Intracellular glucose is metabolized into UDP-galactose by several enzymes including hexokinase 1 (HK1), phosphoglucomutase (PGM), and UDP-glucose pyrophosphorylase (UGP). Glucose and UDP-galactose are then transferred into the Golgi apparatus via GLUT1 and SLC35A2, respectively. MECs finally synthesize lactose from glucose and UDP-galactose by β -1,4-galactosyltransferase (β 4Galt) binding with α -lactalbumin (α -LA) [4]. α -LA is a MEC-specific protein that changes the enzyme specificity of β 4Galt to synthesize lactose by binding to a specific site of β 4Galt during lactation [5]. Without the binding of α -LA, β 4Galt synthesizes Gal- β -1-4-GlcNAc-disaccharide but not lactose. Therefore, lactose is synthesized only by MECs expressing α -LA [6, 7].

In normal mammary glands, MECs initiate lactose synthesis after parturition and maintain its synthesis during lactation [1, 8]. However, the synthesis of lactose is downregulated by mastitis, the inflammation of mammary glands owing to bacterial infection with pathogens such as *Staphylococcus aureus*, coagulase-negative staphylococci, and *Escherichia coli* [9-12]. Infected mammary glands show a decrease in milk yield and compositional changes in milk, particularly with respect to lactose concentration. For example, in sheep, experimental infection with *Staphylococcus aureus* and *Staphylococcus simulans* causes a decrease in the concentration and total yield of lactose in milk, whereas it increases the milk's protein and fat content [12, 13]. The percentage of lactose in milk is reduced after challenge with *Streptococcus uberis*, whereas the percentages of fat and protein in milk does not differ between infected and uninfected cows [14]. Intramammary challenge with *Escherichia coli* and lipopolysaccharide (LPS) in dairy cows during lactation reduces both the concentration of lactose in the milk and the total yield of lactose after infection to a greater extent than that observed with fat [9, 15]. Furthermore, the lactose concentration in the glands infected with coagulase-negative staphylococci is significantly lower than that in the uninfected ones, although the whey protein and albumin concentrations are significantly

higher in the infected glands [16]. Additionally, we have previously reported that the lactose synthesis pathway is markedly down-regulated in LPS-induced mouse mastitis [17]. These reports suggest that the lactose synthesis pathway is affected by mammary gland inflammation caused by bacterial infection.

Infected mammary glands attempt to eliminate pathogens through multiple immune responses including the release of inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 [18, 19]. However, inflammatory cytokines may cause a decrease in milk production in MECs. For example, TNF- α has been reported as a regulator of apoptosis during involution [20, 21]. IL-1 β and TNF- α are cytokines that activate NF κ B signaling, which downregulate β -casein expression in MECs [22]. IL-6 expression increases at the onset of involution, and loss of IL-6 results in delayed mammary gland involution [23]. Inflammatory cytokines are thought to cause adverse effects directly in MECs. However, it remains unclear how TNF- α , IL-1 β , and IL-6 directly influence the lactose synthesis pathway in MECs.

There are several types of *in vitro* lactation models for research on lactating MECs [24-29]. In this study, we prepared two types of *in vitro* murine MEC culture models. One is a monolayer culture of MECs treated with prolactin, dexamethasone (DEX), insulin, and epidermal growth factor (EGF) to induce milk secretion in undifferentiated MECs. Second is the three-dimensional culture of MECs surrounded by Matrigel, which is a basement membrane matrix extracted from Engelbreth-Holm-Swarm mouse sarcoma cells. MECs cultured in Matrigel form alveolar structures with clear apical-basal polarity of the epithelial cells and alveolar lumen-like space [27]. In this study, these *in vitro* models revealed the direct influences of inflammatory cytokines on lactose synthesis pathway.

Materials and Methods

Animals

Virgin and pregnant female ICR mice were purchased from Japan SLC Inc., (Shizuoka, Japan) and were maintained under conventional conditions at 22–25°C. After parturition, the lactating mice were kept with at least ten suckling neonatal pups. The mice were decapitated and the fourth mammary glands were excised for isolation of the epithelial fragments and MECs. All experimental procedures in this study were approved by the Animal Resource Committee of Hokkaido University and were conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

Reagents

Prolactin from sheep pituitary, DEX, and insulin were purchased from Sigma-Aldrich (St. Louis, MO). RPMI-1640 medium, fetal bovine serum (FBS) and antibiotics were from GIBCO-BRL (Grand Island, NY). Type III collagenase and EGF were obtained from Worthington Biochemical Corporation (Lakewood, NJ) and BD Biosciences (Bedford, MA), respectively. TNF- α , IL-1 β , and IL-6 were purchased from PeproTech Inc. (Rocky Hill, NJ).

The following antibodies served as primary antibodies: rabbit polyclonal antibodies against STAT5 (Cell Signaling Technology, Danvers, MA, # 9363, 1:1000), phosphorylated-STAT5 (pSTAT5; Cell Signaling Technology, # 4322, 1:500), pSTAT5a (Abcam, # ab30648, 1:200), NF κ B (Abcam, Cambridge, UK, # ab7970, 1:1000), pNF κ B (Cell Signaling Technology, # 3033, 1:500), and GLUT1 (Dako, Carpinteria, CA, #A3536, 1:200); mouse monoclonal antibodies against pan-keratin (Sigma-Aldrich, # C2562, 1:200); guinea pig polyclonal antibody against adipophilin (Progen, Heidelberg, Germany, #GP40, 1:500); and a goat polyclonal antibody against β -casein (Santa Cruz Biotechnology, #sc-17969, 1:200). The secondary antibodies (an Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody, Alexa Fluor 546-conjugated goat anti-mouse IgG antibody, Alexa Fluor 488-conjugated goat anti-guinea pig IgG antibody, and Alexa Fluor 546-conjugated donkey anti-goat IgG antibody) were purchased from Life Technologies (Gaithersburg, MD). The secondary horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Sigma-Aldrich.

Cell culture

MECs were isolated from the fourth mammary gland of virgin ICR mice. The minced mammary glands were incubated with the RPMI-1640 medium containing type III collagenase at 1.5 mg/mL for 2 h at 37°C with shaking at 70 rpm, followed by treatment with 0.25% trypsin in RPMI-1640 for 5 min at room temperature. After centrifugation, the pellet was resuspended in 60% FBS in RPMI-1640 medium and then centrifuged at 5 $\times g$ for 5 min to separate the epithelial fragments from single cells including adipocytes, macrophages, and myoepithelial cells. The epithelial fragments were seeded on the culture dish with growth medium containing RPMI-1640 containing 10% FBS, 10 μ g/mL insulin, 10 ng/mL EGF, 100 U/mL penicillin, and 100 μ g/mL streptomycin. For immunofluorescence staining, MECs were cultured on a poly-L-lysine-coated glass coverslip. After the MECs spread outwards from the epithelial fragments and reached confluence, the culture

medium was changed to the differentiation medium: RPMI-1640 containing 1% FBS, 10 µg/mL insulin, 10 ng/mL EGF, 0.5 U/mL prolactin, and 10 nM DEX. TNF-α, IL-1β, and IL-6 were added into the differentiation medium at a final concentration of 20 ng/mL.

The three-dimensional culture of MECs for reconstitution of alveolar structure was performed by the method developed by J. Debnath with some modification [30]. Briefly, the mammary epithelial fragments were suspended with growth medium containing 2% Matrigel® (Growth Factor Reduced, BD, #354230) and were cultured for 5 days on the solid Matrigel with growth medium containing 2% Matrigel. After the MECs formed alveolar-like structures, the medium was changed to the differentiation medium containing 2% Matrigel. TNF-α, IL-1β, and IL-6 were added into the differentiation medium at a final concentration of 20 ng/mL.

Immunofluorescence staining

The cells on glass coverslips were fixed with methanol for 10 min at -20°C followed by 1% formaldehyde in PBS for 10 min at 4°C. After treatment with 0.2% Triton X-100 in PBS for 5 min at room temperature, the fixed MECs were incubated with PBS containing 5% bovine serum albumin (BSA; Sigma-Aldrich) to block nonspecific interactions. They were then incubated with the primary antibody diluted in the blocking solution overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBST), the glass coverslips were incubated with the secondary antibodies diluted in the blocking solution for 1 h at room temperature. Control samples were processed in the same manner, with the exception that the primary antibody was absent. Immunofluorescence staining images were obtained with a confocal laser-scanning microscope (TCS SP5; Leica, Mannheim, Germany).

Western blotting

The samples of MECs were electrophoresed using a 7.2% or 12.5% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h with PBST containing 3% nonfat dried milk and then incubated overnight at 4°C with primary antibodies diluted in PBST containing 5% BSA. Subsequently, the membranes were washed in PBST and incubated for 45 min at room temperature with the appropriate secondary horseradish peroxidase-conjugated antibody diluted in PBST containing 3% nonfat dried milk. The immunoreactive bands were detected using Luminata Forte Western HRP substrate (Millipore, Billerica, MA). The

images of the protein bands were obtained with a Bio-Rad ChemiDoc™ EQ densitometer and Bio-Rad Quantity One® software (Bio-Rad).

Reverse transcription PCR

Total RNA from the mammary glands and MECs was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription (RT) was performed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). RT-PCR was conducted on a Life Touch thermal cycler (Life Eco, Bioer Technology, Hangzhou, China) with the KAPA Taq Extra HS Ready Mix (KAPA Biosystems, Wilmington, MA). We used the following cycling conditions: 95°C for 3 min, followed by 30-40 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 20 s. The primer sequences are listed in Table 1.

The quantitative RT-PCR was conducted on a Light Cycler 480 (Roche Applied Science, Indianapolis, IN) with the Thunderbird SYBR qPCR Mix (Toyobo). We used the following cycling conditions: 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 56°C for 1 min. *Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* served as an internal control.

Statistical analysis

Data are expressed as mean values (S.E.). The statistical significance of differences between mean values was evaluated using Student's t test. Differences were considered significant at p-values of <0.05 and <0.01, indicated by asterisks. All experiments were performed a minimum of four times using MECs originated from different culture dishes.

Results

Milk-producing ability of MECs

For preparation of MECs, mammary epithelial fragments were isolated by digestion of the murine mammary glands with collagenase and were seeded on the culture dish (Fig. 1A). MECs were radially extended from the epithelial fragments after 1 day of culture and reached confluence within 6 days of culture (Fig. 1B, C). Subsequently, MECs were treated with the differentiation medium containing prolactin, EGF, DEX, and insulin to induce lactogenesis. MECs treated with differentiation medium showed nuclear localization of the phosphorylated form of STAT5 (Fig. 1D). STAT5 is the transcription factor for lactation, and activation of

the STAT5 pathway is confirmed by translocation of phosphorylated STAT5 into nuclei [31]. The milk-specific β -casein was localized in MECs (Fig. 1E). Adipophilin, which is a major protein that coats milk lipid globules [32], was also localized to the surface of lipid droplets in MECs (Fig. 1F). The mRNA expression of lactose synthesis-related genes was also examined. MECs showed expression of *Glut1*, *Glut12*, *Pgm1*, *Hk1*, *Ugp2*, *Slc35a2*, *B4galt3*, and *Lalba* (α -LA) similar to that observed in lactating mammary glands and the epithelial fragments (Fig. 1G). These results suggest that MECs treated with differentiation medium have the ability to produce milk components.

Expression pattern of cytokine receptors in MECs

Expression of cytokine receptors in MECs was investigated by RT-PCR. *Tnfr1* and *Tnfr2* for TNF- α , *Il1r1* and *Il1r2* for IL-1 β , and *Il6st* and *Il6ra* for IL-6 were expressed in MECs in a manner similar to that in lactating mammary glands *in vivo* and the isolated epithelial fragments (Fig. 2A). Localization of TNFR1 and IL1RL1 was also observed in MECs by immunofluorescence staining (Fig. 2B, C). These results suggest that MECs have the ability to respond to TNF- α , IL-1 β , and IL-6. In fact, MECs treated with inflammatory cytokines changed their arrangements 1 day after treatment (Fig. 3). In the absence of inflammatory cytokines, MECs showed a tightly arranged cobblestone appearance. MECs treated with TNF- α and IL-1 β gradually got rounder with an increase in intercellular space. On the other hand, IL-6 treatment did not cause any detectable differences in MECs at least not at 1 day and 3 days post-treatment.

Different influences of inflammatory cytokines on lactose synthesis-related genes

The mRNA expression level of lactose synthesis-related genes was measured by quantitative RT-PCR after treatment with inflammatory cytokines. TNF- α significantly decreased the expression levels of *Glut12*, *Pgm1*, *Hk1*, *Ugp2*, *Slc35a2*, *B4galt3*, and α -La in MECs at 1 and/or 3 days after treatment, although the expression level of *Glut1* changed negligibly (Fig. 4A-H). In particular, the expression level of α -La at 1 day after TNF- α treatment was about one-thirtieth compared to that of the control. Three days after IL-1 β treatment, the expression level of *B4galt3* was significantly reduced by almost half of that in non-treated MECs. The expression level of *Glut1* and α -La also showed downward tendencies with IL-1 β treatment. IL-6 significantly increased the expression levels of *Glut1* and *Slc35a2*, whereas it decreased the expression levels of *Hk1* and *Glut12* in MECs at 1

day after treatment.

Internalization of GLUT1 by treatment with TNF- α and IL-1 β

GLUT1 is a glucose transporter localized in the basolateral membrane of MECs in normal lactating mammary glands [2]. However, internalization of GLUT1 from the cell membrane to the cytoplasm occurs in mastitis models by injection of LPS [33].

In MECs without cytokine treatment, GLUT1 was mainly localized in the lateral membranes but not in the cytoplasm or Golgi apparatus (Fig. 5A). In MECs treated with TNF- α , GLUT1 was mainly localized in the cytoplasm but not the lateral membranes (Fig. 5B). Similar localization of GLUT1 was also observed in MECs treated with IL-1 β (Fig. 5C). IL-6 treatment caused partial translocation of GLUT1 from the lateral membrane to the cytoplasm (Fig. 5D). Western blotting analysis showed a drastic decrease in the GLUT1 bands at 42 and 50 kDa (probably the glycosylated form of GLUT1) by TNF- α and IL-1 β treatment, although IL-6 did not change the band pattern of GLUT1 compared to that of the control (Fig. 5E).

To clearly observe the localization of GLUT1 in the basal membranes of MECs treated with cytokines, we prepared reconstructed alveoli by three-dimensional culture of the mammary epithelial fragments on Matrigel. The epithelial fragments gradually got rounder and formed luminal spaces in the presence of Matrigel (Fig. 6A-C). In the reconstituted alveoli, GLUT1 was mainly localized in the basal membranes (Fig. 6D). However, the alveolar-like epithelium treated with TNF- α and IL-1 β showed a decrease in GLUT1 in the basal membranes (Fig. 6E, F). Moreover, intracellular GLUT1 was observed in the alveolar-like epithelium with IL-1 β and IL-6 treatments (Fig. 6F, G).

STAT5 and NF κ B in MECs treated with inflammatory cytokines

To understand the influence of inflammatory cytokines in the activation of STAT5 and NF κ B in MECs, their phosphorylation status and nuclear localization were investigated by western blotting and immunofluorescence staining, respectively. TNF- α and IL-1 β treatment increased phosphorylated NF κ B levels, whereas IL-6 treatment did not show any detectable difference (Fig. 7A). Nuclear localization of NF κ B was also observed in immunofluorescence images of MECs treated with TNF- α and IL-1 β (Fig. 7B).

Level of phosphorylated STAT5 was decreased by TNF- α treatment, whereas that of non-phosphorylated STAT5 was almost the same between MECs treated

with vehicle (PBS; control), TNF- α , IL-1 β , and IL-6 (Fig. 7A). Positive reactions to phosphorylated STAT5a in the nuclei of MECs were decreased by TNF- α treatment and IL-1 β decreased the number of pSTAT5a-positive cells (Fig. 7B). On the other hand, MECs treated with IL-6 did not show any obvious differences in phosphorylation, as determined by western blotting analysis, or in localization of STAT5, as determined by immunofluorescence staining, compared to that observed in the control.

Discussion

In this study, MECs isolated from murine mammary glands were cultured on a culture dish or in Matrigel in differentiation medium containing prolactin and DEX for the preparation of *in vitro* culture models of lactation. MECs cultured on the dish showed activation of STAT5 and expression of β -casein and adipophilin. STAT5 is the transcription factor responsible for the milk-producing ability of MECs [34]. Beta-casein is one of the major milk proteins, and adipophilin localizes on the surface of cytoplasmic lipid-droplets in MECs [32, 35]. GLUT1, GLUT12, PGM1, HK1, UGP2, SLC35A2, B4GALT3, and α -LA are highly expressed in lactating mammary glands after parturition and facilitate lactose synthesis during lactation [1]. MECs treated with the differentiation medium expressed these lactose synthesis-related genes. MECs also showed expression of the receptors for TNF- α (*Tnfr1*, *Tnfr2*), IL-1 β (*Il1r1*, *Il1r2*), and IL-6 (*Il6st*, *Il6ra*). These results indicate that MECs treated with the differentiation medium have the ability to produce milk and show reactivity to inflammatory cytokines similar to those in lactating mammary glands. In addition, MECs surrounded by Matrigel showed reconstitution of the alveolar structure with epithelial cell polarity similar to those in lactating mammary glands. This model was suitable for observation of intracellular localization of GLUT1 by immunostaining. Therefore, we used these two types of culture models to investigate the influence of inflammatory cytokines on the lactose synthesis ability of MECs.

TNF- α , IL-1 β , and IL-6 showed differing influences on the lactose synthesis pathway in MECs. TNF- α decreased the expression of *Glut12*, *Pgm1*, *Ugp2*, *Slc35a2*, *B4galt3*, and α -LA in MECs. TNF- α also caused degradation and internalization of GLUT1 from the basolateral membranes into the cytoplasm. GLUT1 and GLUT12 localize in the basolateral and apical membranes, respectively, to take in extracellular glucose [36]. PGM1, HK1, and UGP2 are enzymes required for the interconversion of glucose to UDP-galactose [1]. GLUT1 and *Slc35a2* transport

glucose and UDP-galactose into the Golgi apparatus, respectively. The complex of B4galt and α -LA synthesizes lactose from glucose and UDP-galactose [5]. Thus, TNF- α is suggested to cause multiple adverse effects in the lactose synthesis pathway in MECs. IL-1 β caused partial adverse effects on the lactose synthesis pathway through the internalization of GLUT1 and a decrease in the expression level of *B4galt3*. IL-6 caused both stimulatory and adverse effects on the lactose synthesis pathway in MECs. IL-6 treatment decreased expression of *Glut12* and *Hk1*, whereas it induced the up-regulation of *Glut1* and *Slc35a2*. These observations indicate that TNF- α , IL-1 β , and IL-6 have differing effects on the lactose synthesis pathway in MECs.

The concentrations of inflammatory cytokines increase in milk and blood after intramammary infection in response to invading bacteria as one of the defense mechanisms [37-39]. MECs themselves also secrete inflammatory cytokines including TNF- α , IL-1 β , and IL-6 in response to pathogens and endotoxins in mastitis. For example, *Staphylococcus aureus* and *Escherichia coli* directly induce high expression of inflammatory cytokines including TNF- α , IL-1 β , and IL-6 in bovine MECs [40]. Endotoxins originating from pathogens also induce secretion of inflammatory cytokines [41-43]. Furthermore, mRNA expression of *TNF- α* , *IL-1 β* , and *IL-6* increases in MECs infected with *Staphylococcus aureus* even if antibiotics effective against *Staphylococcus aureus* were used for treatment [44]. Thus, MECs are exposed to these inflammatory cytokines in a wide variety of mastitis situations through paracrine and autocrine mechanisms. The receptors for TNF- α , IL-1 β , and IL-6 were confirmed both in cultured MECs and in lactating mammary epithelium in this study. Therefore, it is suggested that the lactose synthesis pathway in MECs is regulated by TNF- α , IL-1 β , and IL-6 in a wide variety of mastitis situations in conjunction with the stimuli of pathogens and their endotoxins. In particular, TNF- α is a key inhibitory factor of the lactose synthesis pathway in lactating MECs.

NF κ B activity increases during pregnancy, decreases during lactation, and then increases again after weaning [45, 46]. This activation pattern suggests that downregulation of the NF κ B pathway plays an important role during lactation. In fact, NF κ B activation has been shown to result in a more rapid loss of milk and secretory structures in the lactating mammary glands in mice [47]. Activation of NF κ B by TNF- α also inhibits expression of β -casein [48]. In contrast, STAT5, a transcription factor activated by prolactin, maintains the expression of genes related to milk production in MECs during lactation [34]. However, endotoxins cause inactivation of the STAT5 pathway [49]. We have previously reported that

LPS causes down-regulation of the lactose synthesis pathway in association with activation of NFκB and inactivation of STAT5 [33]. In this study, MECs treated with TNF-α showed activation of NFκB and inactivation of STAT5, suggesting that NFκB and STAT5 signaling pathways are involved in the multiple adverse effects caused by TNF-α in the lactose synthesis pathway. In addition, IL-1β induced the activation of NFκB, while the activation of STAT5 was unaffected. In addition, MECs changed their arrangements after treatment with TNF-α and IL-1β in association with activation of NFκB. NFκB activation is known to repress gene expression of E-cadherin and Zonula Occludens-1, which maintain cell-cell adhesion and cell morphology [50-52]. IL-6 had no influence on STAT5 and NFκB. However, IL-6 has been reported to contribute to the remodeling of mammary tissue during involution through the MAPK and STAT3 signaling pathways [23]. In this study, IL-6 induced the down-regulation of *Glut12* and *Hk1* and the up-regulation of *Glut1* and *Slc35a2*. IL-6 is supposed to influence on these lactose synthesis-related genes through STAT3 pathway. The differences in intracellular signaling pathways activated by each inflammatory cytokine may be the reason for their differing effects on the lactose synthesis pathway. Furthermore, inflammatory cytokines may interfere with differentiation process of MECs because MECs were exposed to the inflammatory cytokines without pretreatment with the normal differentiation medium containing prolactin and DEX.

In summary, our results show that TNF-α, IL-1β, and IL-6 have different effects on the lactose synthesis pathway in MECs. In particular, TNF-α causes down-regulation of lactose synthesis-related genes concurrently with degradation of GLUT1 from the basolateral membrane in MECs. TNF-α, IL-1β, and IL-6 levels increase in lactating mammary glands in a wide variety of mastitis situations and MECs are, unsurprisingly, exposed to them [37-39]. Therefore, we suggest that the lactose synthesis pathway in MECs is regulated by these inflammatory cytokines in conjunction with pathogens and their endotoxins. STAT5 and NFκB are thought to be involved in the adverse effects of TNF-α. However, the intracellular signaling pathways to regulate lactose synthesis in mastitis are thought to be overly complicated because each of the cytokines and pathogens has been reported to activate several signaling pathways [53-55]. Further investigation is required to elucidate the intricate regulatory mechanisms of lactose synthesis in the lactating MECs under conditions of inflammation.

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Figure captions

Figure 1

Cell culture models of lactation, using mammary epithelial cells

(A-C) Phase-contrast images of mammary epithelial fragments isolated from the murine mammary glands (A), extended mammary epithelial cells (MECs) from the epithelial fragments after 1 day of culture (B), and MECs at confluence after 6 days of culture (C). (D-F) Immunofluorescence staining images of phosphorylated STAT5a (pSTAT5a, green, D); pan-keratin (red, D), as a marker for epithelial cells; β -casein, a major milk protein (red, E); and adipophilin of a lipid droplet coating protein (F, green) in MECs treated with the differentiation medium for 3 days. Blue indicates nuclei stained with DAPI. Scale bars: 20 μ m. (G) Band images showing mRNA expression of lactose synthesis-related genes (*Glut1*, *Glut12*, *Pgm1*, *Hk1*, *Ugp2*, *Slc35a2*, *B4galt3*, and *Lalba*) in the lactating mammary glands at 3 days after parturition, epithelial fragments originating from lactating mammary glands, and MECs treated with the differentiation medium for 3 days.

Figure 2

Expression of receptors for inflammatory cytokines in mammary epithelial cells

(A) Band images showing mRNA expression of receptors for TNF- α (*Tnfr1*, *Tnfr2*), IL-1 β (*Il1r1*, *Il1r2*), and IL-6 (*Il6st*, *Il6ra*) in the lactating mammary glands at 3 days after parturition, epithelial fragments originating from lactating mammary glands, and MECs treated with the differentiation medium for 3 days. (B, C) Immunostaining images show the localization of TNFR1 (green, B) and IL1RL1 (green, C) in MECs treated with the differentiation medium for 3 days. Blue indicates nuclei stained with DAPI. Scale bars: 10 μ m.

Figure 3

Phase-contrast images of mammary epithelial cells treated with inflammatory cytokines

Phase-contrast images showing mammary epithelial cells treated with the differentiation medium containing vehicle (PBS), TNF- α , IL-1 β , and IL-6 for 1 and 3 days. The final concentration of each inflammatory cytokines in the medium is 40 ng/ml. Scale bars: 20 μ m.

Figure 4

Influence of inflammatory cytokines on the expression of lactose synthesis-related genes

Relative expression levels of *Glut1* (A), *Glut12* (B), *Pgm1* (C), *Hk1* (D), *Ugp2* (E), *Slc35a2* (F), *B4galt3* (G), and *Lalba* (α -LA; H) in mammary epithelial cells at 1 day (n = 4) and 3 days (n = 5) after treatment with inflammatory cytokines at a final concentration of 40 ng/ml were quantified by real-time PCR. Data represent the mean (S.E.). *p < 0.05 vs. control.

Figure 5

Influence of inflammatory cytokines on the localization and expression of GLUT1 in mammary epithelial cells

(A-D) Immunofluorescence staining images of GLUT1 (green) in mammary epithelial cells treated with the differentiation medium containing vehicle (PBS; A), TNF- α (B), IL-1 β (C), and IL-6 (D) at a final concentration of 40 ng/ml for 3 days. Blue indicates nuclei stained with DAPI. Scale bars: 20 μ m. (E) Band images by western blot analysis showing the expression of GLUT1 and β -actin in mammary epithelial cells treated with inflammatory cytokines at 40 ng/ml for 3 days.

Figure 6

Localization of GLUT1 in a three-dimensional culture of mammary epithelial cells treated with inflammatory cytokines

(A-C) Phase-contrast images showing the mammary epithelial fragments on the Matrigel after 0 (A), 3 (B), and 6 days (C) of culture. MECs of the epithelial fragments gradually formed alveolar-like structures after cultivation. Scale bars: 50 μ m. (D-G) Immunofluorescence staining images of GLUT1 (green) in the alveolar-like structures after treatment with the differentiation medium containing vehicle (PBS; D), TNF- α (E), IL-1 β (F), and IL-6 (G) for 1 or 3 days. Blue indicates nuclei stained with DAPI. Scale bars: 10 μ m.

Figure 7

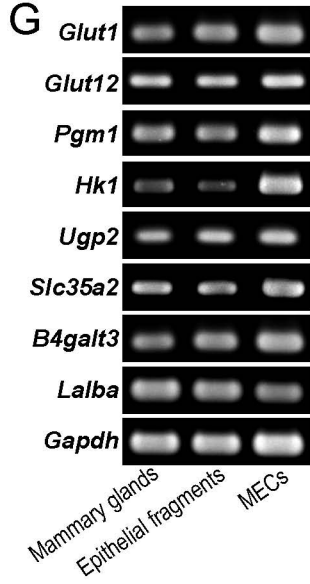
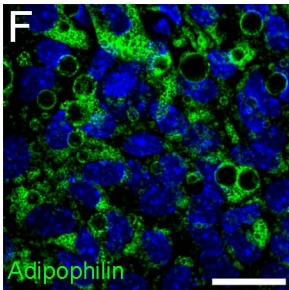
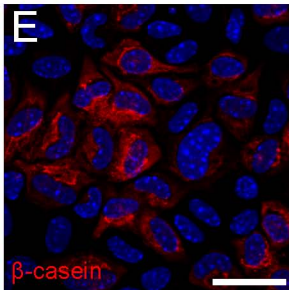
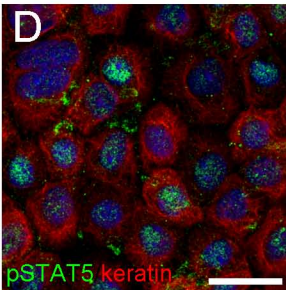
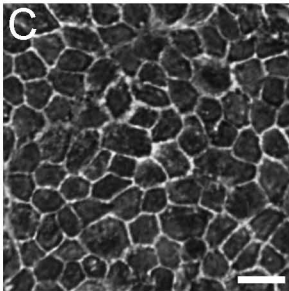
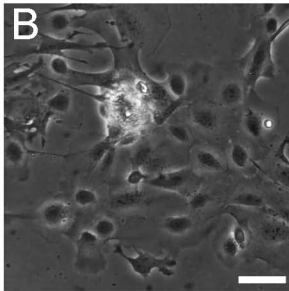
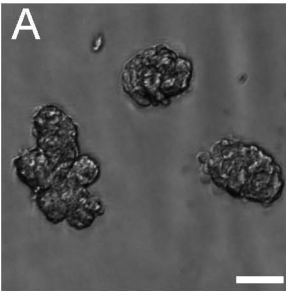
Influence of inflammatory cytokines on activation of STAT5 and NF κ B in mammary epithelial cells

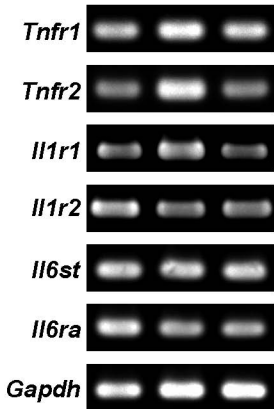
(A) The phosphorylation of STAT5 and NF κ B was examined by western blotting analysis of mammary epithelial cells (MECs) treated with vehicle (PBS), TNF- α , IL-1 β , and IL-6 for 1 day by using antibodies against STAT5, phosphorylated STAT5 (pSTAT5), NF κ B, phosphorylated NF κ B (pNF κ B), and β -actin. (B)

Immunofluorescence staining images of NF κ B (green) with pan-keratin (red) and pSTAT5a (green) of MECs treated with vehicle (PBS), TNF- α , IL-1 β , and IL-6 for 1 day. Blue indicates nuclei stained with DAPI. Scale bars: 100 μ m.

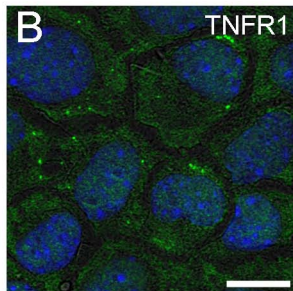
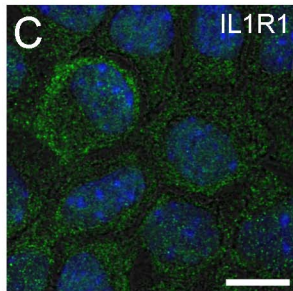
Table 1
Primer Sequences for RT-PCR

Gene	Accession number	Primers		product size
		Forward	Reverse	
<i>Glut1 (Slc21a)</i>	NM_011400	gcttctgctcatcaatcgt	gccgaccctcttctttcatc	117
<i>Glut12 (Slc2a12)</i>	NM_178934.4	atcgctggcttttgtgttct	gctctggggctttcgttt	170
<i>Pgm1</i>	NM_025700.2	aggagggcaaaggtgtcttg	cactctccactcaccactgtc	134
<i>Hk1</i>	NM_001146100.1	ccgagctgaaggatgaccaa	ccccttttctgagccgtcc	200
<i>UGP2</i>	NM_139297	tcacaaacaaaacacgagcaga	cacttgagcgatttcacca	89
<i>Slc35a2</i>	NM_001083937.1	ctgtgctggtggtccagaat	caggtgcttcacattaccct	170
<i>B4galt3</i>	NM_020579.2	aagtttgatacagcctcccg	tcccacagaggtaggtggtc	186
<i>α-lactalbumin (Lalba)</i>	NM_010679	accagtggctacgacacac	cggggaactcactacttttacac	106
<i>Tnfr1</i>	NM_011609.4	agccacaccacacaccttag	tttcacccacagggagtagg	80
<i>Tnfr2</i>	NM_011610.3	tgtagaggggaaggaacacg	cacaccaggaacagtcctt	106
<i>Il1r1</i>	NM_008362.2	gagttacccgaggtccagtgg	gagggctcaggataacagg	235
<i>Il1r2</i>	NM_010555.4	ggtgcggaacaatgttcatttg	gggaactgctggagatctcggagtg	239
<i>Il6st</i>	NM_001008725.3	aggaattggctgagcgccgtt	aggcactgtgtggttaaccgctat	114
<i>Il6ra</i>	NM_001310676.1	gtggatgttccccagaggagcc	aacagcacagccttcgtggtt	119
<i>Gapdh</i>	NM_008084	gagcgagaccccactaacatc	gcggagatgatgaccctttt	144



A

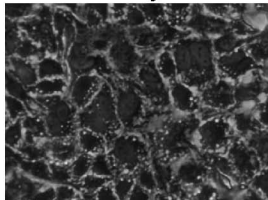
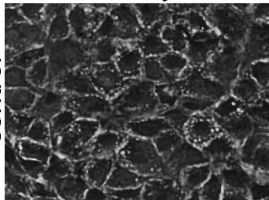
Mammary glands
Epithelial fragments
MECs

B**C**

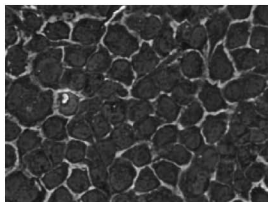
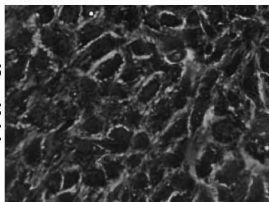
1 day

3 days

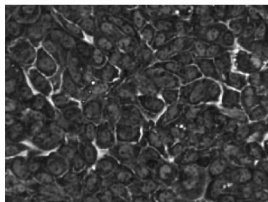
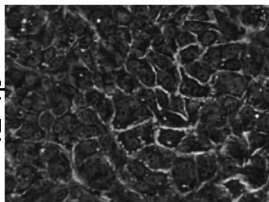
control



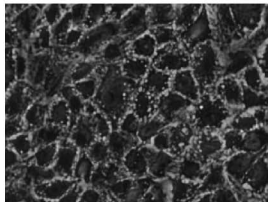
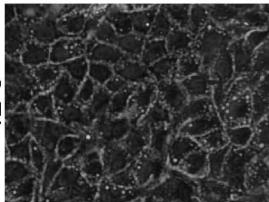
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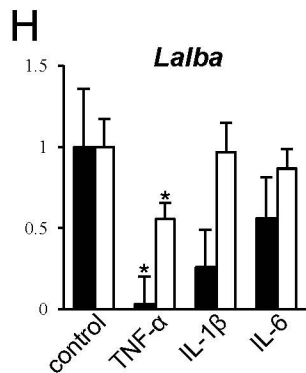
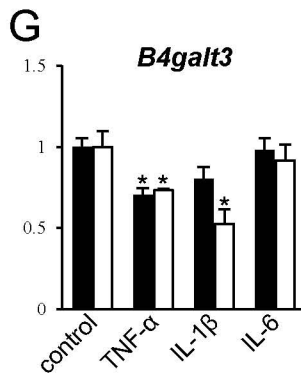
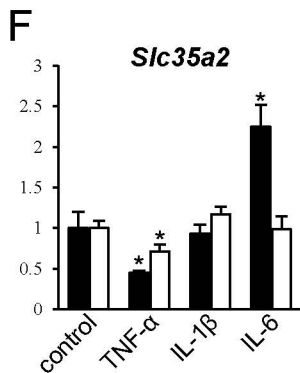
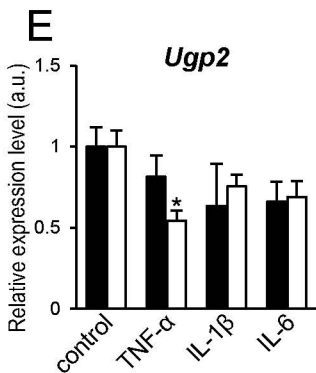
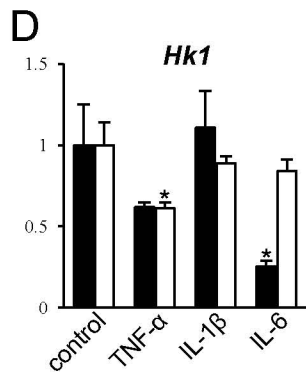
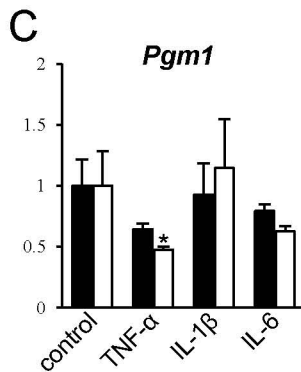
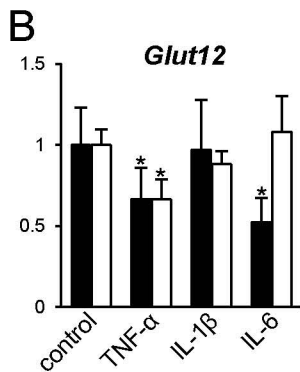
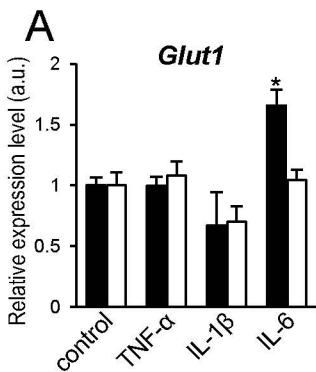


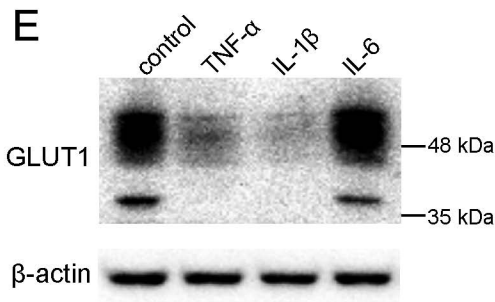
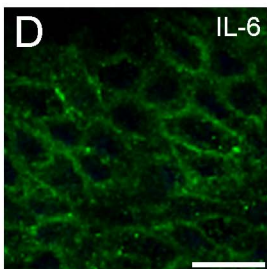
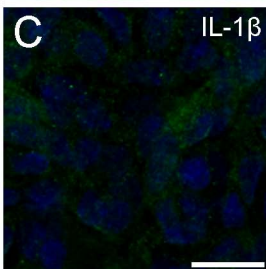
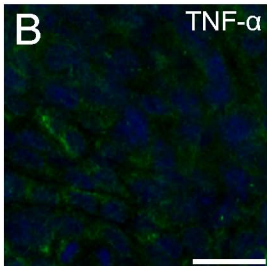
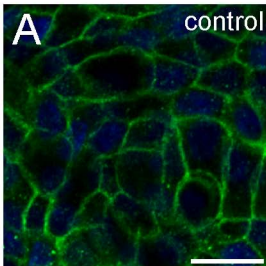
IL-1 β

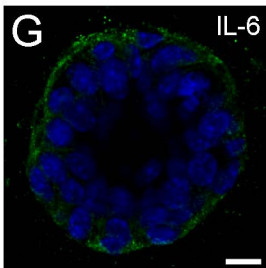
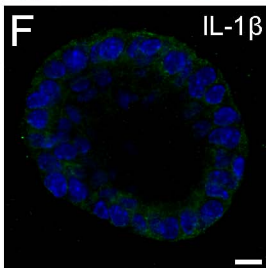
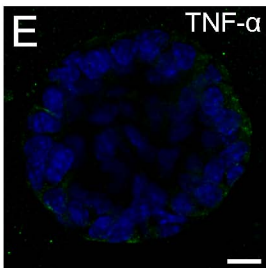
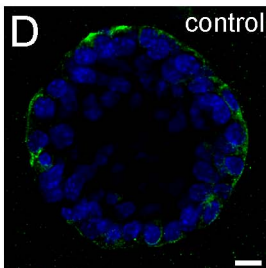
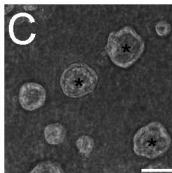
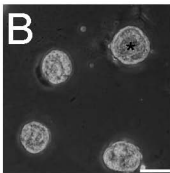
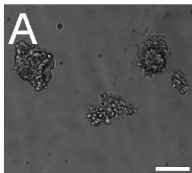


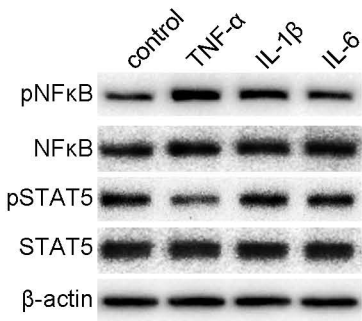
IL-6









A**B**