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Expression of acute phase proteins and inflammatory cytokines in mouse mammary gland following *Staphylococcus aureus* challenge and in response to milk accumulation

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We used a mouse model of pathogenic (*Staphylococcus aureus*) and non-pathogenic (teat sealing) mammary inflammation to investigate mRNA expression of several inflammatory cytokines and acute phase proteins (APP) in mammary tissue and liver, and the appearance of some of these factors in plasma and milk. The expression levels of IL1β and TNFα were markedly up-regulated in *Staph. aureus*-inoculated mammary tissue at 72 h, whilst IL6 was up-regulated to a lesser extent in a way which was not confined to the inoculated glands. APP expression was up-regulated at 48 and 72 h in both *Staph. aureus*-inoculated and teat-sealed *mammary* glands. These differences between cytokine and APP expression provide additional support for the contention that APPs are produced within the mammary tissue itself during inflammation, rather than in associated immune cells. We propose that measurement of cytokines and APP in combination might provide a tool for diagnostic discrimination between mastitis caused by pathogenic invasion and milk accumulation, and hence allow for better targeting of antibiotic therapy. In comparison with mammary expression, expression of cytokines in liver tissue was up-regulated to a similar or lesser extent, whilst expression of APP was up-regulated to a much greater extent. The first appearance of increased cytokine and APP concentrations in plasma and of milk amyloid A (MAA) in milk occurred in advance of the measurable up-regulation of expression, hence their origin cannot be stated with certainty.

Keywords: Mouse, mammary gland, APP, cytokines, *Staphylococcus aureus*, mastitis.

Pathological inflammation of the mammary gland, mastitis, is one of the most costly diseases in the dairy industry, causing both direct and indirect losses (Seegers et al. 2003; Bannerman, 2009; Cha et al. 2011). The pathogenicity and involvement of *Staphylococcus aureus* as a causative factor in intramammary infection (IMI) has been widely discussed (Bayles et al. 1998; Hensen et al. 2000; Schukken et al. 2011) as has its relevance as a major cause of chronic subclinical mastitis. Defining and establishing better and more reliable diagnostic biomarkers for earlier detection of clinical mastitis would be immensely valuable (Eckersall 1995; Eckersall & Bell, 2010) but so would better methods for diagnosing subclinical mastitis (Akerstedt et al. 2011).

The mammary gland is also subject to inflammatory responses not attributable to microbial invasion. This is most evident at the end of lactation when milk accumulation causes increased intramammary pressure leading to an inflammatory acute phase response (Stein et al. 2004), disruption of the mammary epithelium, apoptosis and extensive tissue remodelling (Quarrie et al. 1996; Theil et al. 2005). Non-pathogenic mammary inflammation may also occur to some extent during the normal filling of the gland between milkings/sucklings, especially if the latter is delayed for some reason (Lakic et al. 2011). In many cases the aetiology of mammary inflammation is not easy to determine. For instance, many breastfeeding practitioners consider that human mastitis rarely involves a pathogen, but is due to 'blocked ducts' and hence milk accumulation (Kvist, 2013). The use of antibiotic therapy to treat human mastitis varies greatly from country to country (Scott et al.

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2008) whereas the majority of bovine mastitis is treated by antibiotic, often without direct evidence of infection (and hence unnecessarily: Roberson, 2012). There is a diagnostic need across species to be able to distinguish between infectious mastitis and 'physiological inflammation'.

The involvement of different cytokines and acute phase proteins (APPs) has been reported in different inflammatory responses including mastitis (see Murata et al. 2004 for review). Tissue damage, including mastitis, induces an acute phase response (APR) which mostly comprises the release of inflammatory mediators from tissue macrophages at the damaged site (Koj, 1996). As a result, mastitic milk contains elevated levels of cytokines (Peli et al. 2003). APPs are also found in milk. Colostrum was shown to contain high levels of mammary-associated serum amyloid A3 (SAA3: McDonald et al. 2001) which was later also found, together with haptoglobin (HP) in naturally occurring and experimentally induced mastitis (Nielsen et al. 2004; Eckersall et al. 2006). A third APP, α1-acid glycoprotein (AGP) is also present in colostrum and mastitic milk (Ceciliani et al. 2005; Eckersall et al. 2007). Systemic as well as local production of APPs is believed to play an important role in host defence (see Eckersall& Bell, 2010 for review).

Using mice as a model for infectious mastitis has been well established for several decades (Chandler, 1970; Anderson, 1977) and more recently has been used for *Staph. aureus* mastitis (Brouillette & Malouin, 2005a; Brouillette et al. 2005b; Hu et al. 2010). However, the murine APR has not been characterised, nor has pathogenic inflammation been compared with non-pathogenic. The objective of this study was to establish a mouse model to allow comparison of the APR in response to infusion of *Staph. aureus* and to accumulation of milk caused by teat sealing. Cytokines included for study were IL1β, IL6 and TNFα, and APPs were AGP, SAA3 and HP. We focused at both local and systemic level, measuring mammary tissue gene expression with qRT-PCR (real-time quantitative PCR) and analysing milk and blood plasma using commercially available ELISA and Multiplex immunoassays. The research addresses two hypotheses: firstly, that acute phase proteins present in milk are in part or wholly of local (mammary) origin; and secondly, that the profile of APP production will differ between pathogenic inflammation and inflammation caused by milk accumulation.

Material and methods

Animals

The experiment was performed according to the guidelines of the European Convention for protection of Vertebrate Animals and Animal Experimentation Act under Danish national legislation and conducted at Technical University of Denmark (DTU), (License Number: 2012-15-2934-00587). NMRI outbred mice (male around 35 g and female around 25 g) were purchased from two different companies, Taconic and Harlan, from Denmark and Netherlands, respectively, in order to establish our own breeding colonies. Experimental mice were fed fortified breeding diet (Altromin Spezialfutter GmbH&Co.KG, Germany) ad libitum. Females were placed singly with males and examined daily for the presence of a vaginal plug. Pregnant mice were transferred into single cages and provided with nesting material. Following parturition the number of pups was adjusted to 10 (Shipman et al. 1987) to ensure a constant suckling stimulus.

Experimental procedure

Lactating mice were used on day 7 ± 2 of lactation. General anaesthesia was induced with isoflurane (IsoFlo Vet, Orion Pharma Animal Health) and continued with hypnorm/dormicum (VetPharma Ltd/Dormicum 5 5 mg/ml ROCHE) 0·075 ml/10 g/mouse. Individual teats were cleaned with 70% ethanol and visualised through a binocular stereo-microscope. A Nanofil™ 100-μl microinjection syringe (World Precision Instruments Inc, SG2 7EG UK) attached to a micromanipulator was carefully positioned such that the tip of a blunt 36-g needle attached to the syringe was close to the tip of the teat, which was then carefully lifted onto the needle using microforceps. Once the needle was located within the teat the contents of the syringe were carefully discharged through the teat into the gland. A total of 36 mice were inoculated in this way. In each mouse, the fourth and fifth inguinal glands on one side were infused with 100 μl of *Staph. aureus* suspension whilst the two contralateral inguinal glands were infused with 100 μl of normal saline vehicle. The second and third thoracic glands on one side were sealed with tissue adhesive (Loctite super glue universal) whilst the contralateral glands were untreated as control. The treatment sides were randomised between mice, and in each mouse the pathogen and sealing treatments were made on opposite sides. Following inoculation the mouse was kept separated from its pups for 4 h in order to obtain better colonisation of the bacteria.

Bacterial strain

Staph. aureus AO35 was obtained from an infected cow and kindly provided by our colleague, Dr Arshnee Moodley, at the Department of Veterinary Disease Biology, Copenhagen University, Denmark. The isolate was propagated in 10 ml of Luria-Bertani (LB) broth for 18 h at 37 °C, sedimented by centrifugation and re-suspended in stile isotonic saline. The viable count was made by counting the number of colonies formed on the LB agar medium, inoculated with 10-μl volumes of tenfold dilution and incubated at 37 °C for 24 h. The suspension was then diluted with sterile isotonic saline to obtain a suspension containing 10 000 cfu/ml. The bacterium was successfully re-isolated from the infused mice (data not shown).

Sampling

The 36 inoculated mice were divided into 6 groups of 6 mice for sample collection at 0 (control), 4, 10, 24, 48 and 72 h

Table 1. Primer sequences used for real-time PCR reactions

Gene	Sequence	Amplicon (bp)	NCBI ref. sequence
IL1β		355	NM_008361.3
Forward	5′GTGTCTTTCCCGTGGACCTT 3′		
Reverse	5′TGGGTGTGCCGTCCTTCATT 3′		
IL6†		453	NM_010551.3
Forward	5′ACACCACTACAGCCCAACAC 3′		
Reverse	5′TTGACCCAGGAGACCAGAAAATC 3′		
SAA3‡		363	NM_011315.3
Forward	5′ AGCCTTCCATTGCCATCATTCTT 3′		
Reverse	5′ AGTATCTTTTAGGCAGGCCAGCA 3′		
HP		180	NM_017370.2
Forward	5′ TGGGGTCCAGCCTATCTTGA 3′		
Reverse	5′ ACACCATACTCAGCGACAGC 3′		
AGP		108	NM_008768.1
Forward	5′ GGAAGCTCAGAACCCAGAACA 3′		
Reverse	5′ CTGAAAGCTGCACCCATGAAA 3′		
TNFα		261	NM_013693.2
Forward	5′AAGAGGCACTCCCCCAAAAG 3′		
Reverse	5′CTTGGTGGTTTGCTACGACG 3′		
Rpl13a		140	NM_009438.5
Forward	5′TTGTGGCCAAGCAGGTACTTC 3′		
Reverse	5′TCGGGAGGGGTTGGTATT 3′		

† IL6 reverse primer sequence was from Wang et al. (2008)
‡ SAA3: Villalba et al. (2012)

after inoculation. Mice were anaesthetised and injected with 0·15 iu oxytocin (Oxytocin-S 0·18 mg/ml, Intervet) IP for milk sample collection into micro-capillary, non-heparinised tubes. Milk samples were obtained from the three treatments (inoculated, vehicle infused and control glands) at 0, 4, 10, 24 and 48 h. Owing to extreme inflammatory response at the bacterial infused gland at the 72-h group samples could not be reliably collected. Blood samples were collected by cardiac puncture and 22-gauge needles, stored at 4 °C overnight then centrifuged at 1500 ***g*** at 4 °C for 10 min for serum collection. Blood sera and milk samples were stored at −80 °C for further analysis. The anaesthetised mouse was then sacrificed by cervical dislocation for tissue sampling. Control, *Staph. aureus*-inoculated, vehicle-infused and teat-sealed mammary tissues were quickly collected and divided into two pieces; a larger portion for flash freezing with liquid nitrogen and storing at −80 °C, and a smaller portion fixed in 4% PFA (v/v) (paraformaldehyde) for 24 h followed by transferring into 70% ethanol for 3 d before paraffin embedding.

RNA preparation

Frozen mammary glands and livers were cut into smaller pieces of 20–25 mg on dry ice. Tissues were homogenised with Trizol (Life Technologies Europe BV, 2850 Naerum, Denmark) and frozen stainless steel beads (5 mm Qiagen Cat.no 69989) with TissueLyser II from Qiagen (Cat.no 85300). They were then centrifuged for 10 min at 4 °C and transferred into MaXtract High Density 1·5-ml tubes (Qiagen Cat.no 129046); final isolation was performed by using SV Total RNA isolation system kit from Promega (Naka, Sweden) according to the guidelines. RNA yield was afterwards checked by NanoDrop ND-1000 Spectrophotometer and the quality of RNA was determined by using Aligent 2100 Bioanalyzer System (Aligent Technologies).

cDNA synthesis, primer design and qRT-PCR

Isolated RNA transcribed to cDNA by using 5× MMLV reverse transcriptase, dNTP, random hexamer primer, RN-ase inhibitor and MMLV enzyme by adding H_2O up to 25 µl cocktail in total (Promega. Nacka, Sweden). The cDNA was stored at −20 °C for short periods and −80 °C for longer times and also diluted to a concentration of 4 ng/µl before quantification.

Three reference genes, RPL13A (ribosomal protein L13a), HPRT (hypoxanthine-guanine phosphoribosyltransferase) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were selected (Han et al. 2010). RPL13A was selected as the most stable reference gene for normalisation of the data. Target and reference genes were tested for genomic DNA on 2% agarose gel, standard curves were obtained, and acceptance efficiency range was set to 1·8–2 with satisfactory melting curves. Products were sent for DNA-sequencing at TAG Copenhagen A/S, Denmark. Samples were run in triplicate with inter-plate calibrators using LighCycler® 480 machine (SYBr green 1 master mix) from Roche Applied and Diagnostics (Hvidovre, Denmark) 10 µM for each primer in a 10-µl reaction mixture containing 2 µl of total cDNA (Rahbek et al. 2014). Primer sequences are listed in Table 1.

Serum cytokine, APP and milk amyloid A concentrations

Serum concentration of IL1β, IL2, IL6, IL10 and TNFα were measured with a Mouse Magnetic Cytokines/Chemokines panel, MCYTOMAG-70 MILLIPLEX Map, Millipore by Luminex 200. The assay was provided with calibration and standards. The observed assay response from calibrators and controls were acceptable and showed the accuracy of the measurements. The samples were run in one batch and all samples were assayed in duplicate.

SAA and HP concentrations were measured by commercially available ELISA kits. Murine serum amyloid A (SAA) (TP-802M), pre-coated microplate and haptoglobin (HP) (TP-801) Tridelta (Development Ltd, Ireland) assays were used for APP measurement in serum samples and by following the manual instruction, both kits were provided with their calibrators. Concentration of mouse milk amlyloid A (MAA) was also determined by using a kit from Tridelta (Development Ltd, Ireland) (TP-807). This ELISA kit was also pre-coated and provided with the calibrators. Concentrations in blood sera and milk samples were determined using BioTek ELx800 Absorbance Microplate Reader® by reading at both 450 and 630 nm, and calculated by Gen5 software. Samples were assayed in duplicate and samples with higher concentration than the standards were re-diluted and reanalysed in order to obtain their concentration values within the range of the standard curves.

Statistical analysis

Relative gene expression analysis was implemented based on Pfaffl (2001) and statistical analysis of all data was performed by using the mixed procedures in SAS (version 9.3, SAS Institute Inc. 2010). Variables in the statistical model included the group, treatment and their interaction as fixed effects, and the factors such as animal, gland side within a row in the animal as random effects, as shown in the following model:

$$Y = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_k + \rho_{lm} + \varepsilon_{ijklm},$$

where Y = the dependent variables, μ = the overall means, α_i = group (i = control, right or left), β_j = treatment (j = control, staph, sealed or saline), $\alpha\beta_{ij}$ = interaction between group and treatment, γ_k = random effect of animal (l = 1, 2,... and 35), ρ_{lm} = random effect of gland side within a row (l = left or right; m = gland number), ε_{ijklm} = random variation which was assumed to be normally distributed with a variance σ^2 and a mean of zero. For expression analysis between control glands and liver samples, variables in the statistical model included the group (control gland and liver), treatment (control, 4, 10, 24, 48 and 72 h) and their interaction as fixed effects, and the factor animal (1, 2,... and 35) as random effect. For analysis of mouse milk amlyloid A (MAA) data, group (control, 4, 10, 24 and 48 h), treatment (control, *Staph.* or saline) and their interaction were included as fixed effect and animal (1, 2,... and 30) as random effect. For analysis of serum amyloid A (SAA) ELISA, colorimetric haptoglobin and mouse MAGNETIC cytokines assays, group (control, 4, 10, 24, 48 and 72 h) was included as fixed effect and animal (1, 2,... and 35) as random effect. Before performing the final statistical analyses, data were checked for outliers based on residual plots. One mouse from the 24-h group was excluded from the whole study.

Presented results are expressed as least squares means ± SEM for the graphs and the significance levels for overall effects in table. The PDIFF option in SAS was used to generate comparisons between treatment means. The level of significance was set at $P < 0{\cdot}05$ and graphs were made with SigmaPlot 12.0.

Results

Mammary gland mRNA expression analysis

Data for mammary gland expression of the three cytokines IL1β, IL6, and TNFα are in Fig. 1 and significance levels for the overall effects of time after inoculation and treatment are in Table 2.

Relative to the reference gene RPL13A, IL1β expression remained at pre-inoculation level until 24 h, when there was a small (non-significant) and transient increase in glands infused with *Staph. aureus*. A much larger and significant ($P < 0{\cdot}05$ *vs.* control) increase occurred in the same treatment at 72 h, whilst all other treatments (including sealed glands) showed no increase (Fig. 1, IL1β). When statistical analysis was restricted to the *Staph. aureus* glands, there was a significant effect of time after inoculation ($P < 0{\cdot}05$) but when all treatments were included there was no significant effect (Table 2). A different pattern was observed for IL6. There was a small but significant ($P < 0{\cdot}05$) increase in overall IL6 expression with time after inoculation, but whilst the eventual increase was numerically greatest in *Staph.* aureus-inoculated glands, there was no significant difference between the treatments at any time point (Fig. 1, IL6). Changes in TNFα were similar to IL1β in that there was a large and significant increase in the *Staph. aureus*-treated glands at 72 h ($P < 0{\cdot}05$ *vs.* control) but in this case there was an overall effect of time after inoculation ($P = 0{\cdot}01$, Table 2) indicating that the increased expression was not restricted to the *Staph. aureus* glands. Nevertheless, there was a significant treatment effect.

Mammary expression of the three APP, HP, AGP and SAA3 are in Fig. 1 (HP, AGP and SAA3). HP was expressed at low levels compared with the cytokines and other APP; nevertheless, there was a clear increase in expression with time after inoculation ($P < 0{\cdot}001$ overall). In contrast to the cytokine patterns, this increase occurred to approximately equivalent extents in *Staph. aureus*- and teat-sealed glands, where it was evident at both 48 and 72 h. At 72 h there were small increases in expression in control and vehicle glands, but the difference between treatment groups was significant ($P < 0{\cdot}05$). Expression of AGP was significantly affected by time after inoculation ($P < 0{\cdot}001$) and by treatment ($P < 0{\cdot}01$) with the main effect being a parallel large increase in

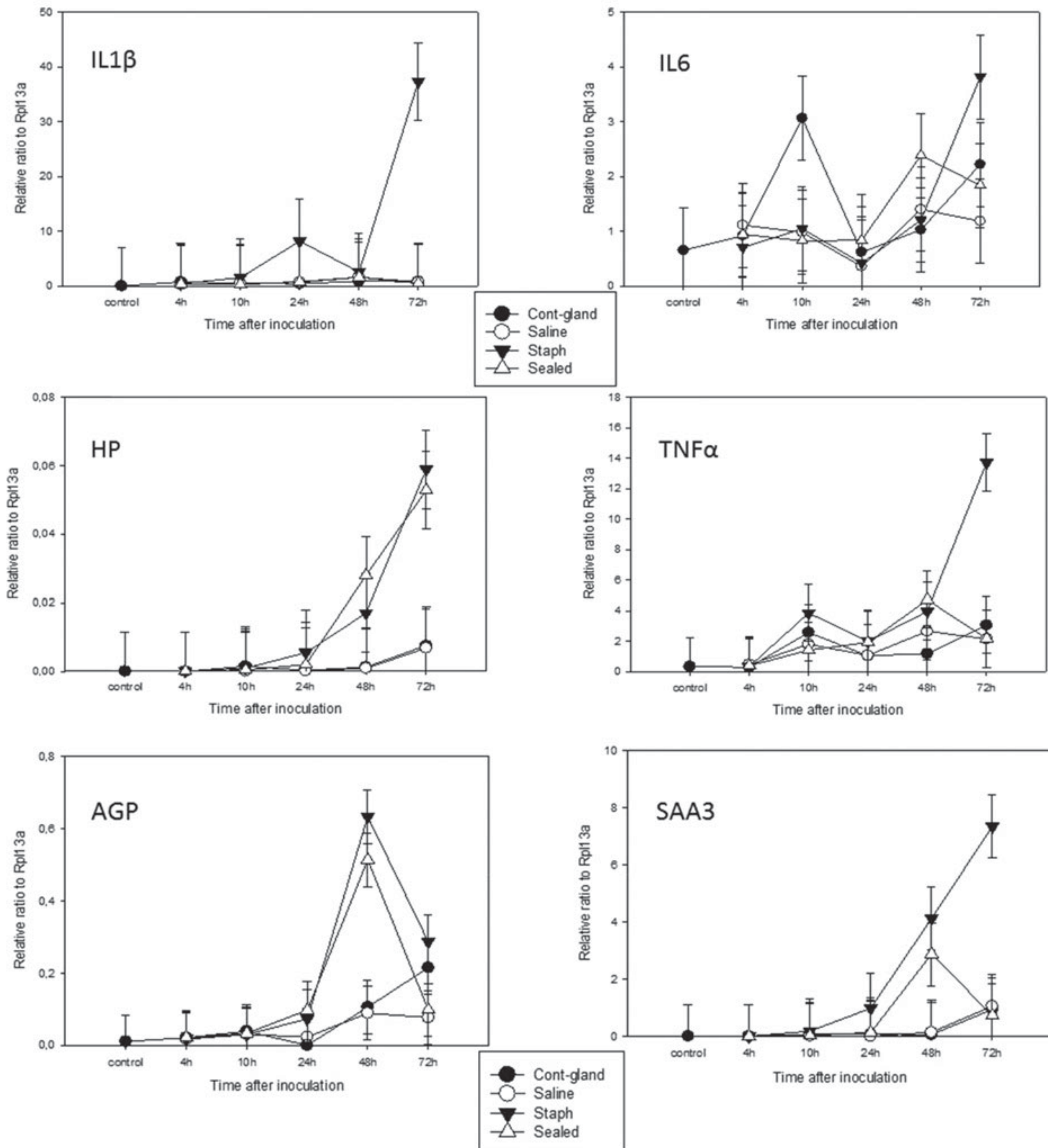

Fig. 1. Relative expression of IL1β, IL6, TNFα, HP, AGP and SAA3 ± SE between four treatments within time intervals for mammary glands.

Staph. aureus- and teat-sealed glands at 48 h, followed by a decrease. There was a clear tendency for expression to increase at 72 h in control and vehicle glands; hence, there was a significant interaction between time and treatment effects ($P<0{\cdot}001$). Expression of SAA3 also increased with time after inoculation ($P<0{\cdot}01$ overall) and in response to treatments ($P<0{\cdot}01$ overall). Initially, there was a parallel increase in *Staph. aureus*- and teat-sealed glands at 48 h, but in the former this increase was sustained to 72 h whereas in the latter there was a decline after 48 h.

Liver mRNA expression analysis

Expression levels of cytokines and APP in liver and control mammary gland tissue are compared in Fig. 2. Based on expression relative to RPL13a, expression of the three cytokines was higher or similar in mammary tissue compared with liver, whilst expression of the three APPs was much lower in mammary gland compared with liver. Hepatic expression of all three cytokines and all three APPs increased significantly with time after inoculation (Table 2). The time

Table 2. Significance levels for overall effects. Data are shown in Figs. Figs. 1–3 and the statistical models used are described in the text. Mammary gland expression of cytokines and APP was analysed for effects of time after inoculation/teat sealing and for treatment effects comparing *Staphylococcus aureus* inoculation, vehicle infusion, teat sealing and no treatment. Liver expression and appearance of cytokines and APP in plasma and milk were analysed only for time after inoculation/teat sealing. NS: not significant. nd: not determined

	Mammary gland expression			Liver expression	Plasma concentration	Milk concentration
	Time	Treatment	Interaction	Time		
IL1β	NS	NS	NS	$P<0{\cdot}05$	nd	nd
IL6	$P<0{\cdot}05$	NS	NS	$P<0{\cdot}05$	$P<0{\cdot}001$	nd
TNFα	$P=0{\cdot}01$	$P<0{\cdot}05$	NS	$P<0{\cdot}01$	nd	nd
HP	$P=0{\cdot}001$	$P=0{\cdot}05$	NS	$P<0{\cdot}001$	$P<0{\cdot}001$	nd
AGP	$P<0{\cdot}001$	$P<0{\cdot}01$	$P<0{\cdot}001$	$P<0{\cdot}001$	nd	nd
SAA3/MAA	$P<0{\cdot}01$	$P<0{\cdot}01$	NS	$P<0{\cdot}001$	$P<0{\cdot}001$	$P<0{\cdot}05$

347 course of increased hepatic expression of IL6 and TNFα was
348 parallel in liver and control mammary tissue, whereas IL1β
349 increased at 72 h in liver only. Hepatic expression of HP,
350 AGP and to a lesser extent SAA3 increased at 48 h, and all
351 three increased further at 72 h.

352 *Blood serum and milk analysis*

353 IL6, HP and SAA serum concentrations were quantified
354 using a cytokine panel and commercially available ELISA
355 kits. Data are in Fig. 3 and significance levels in Table 2. All
356 three markers gradually and significantly increased begin-
357 ning after 10 h. IL6 concentrations were very much lower
358 than either of the APPs, with HP present at much higher
359 levels than SAA. MAA was detected in milk from *Staph.*
360 *aureus*-inoculated glands at 24 and 48 h and from vehicle-
361 infused glands at 48 h, although at levels approximately
362 5-fold lower than in inoculated glands.

363 Discussion

364 Using a novel model that compares pathogenic and non-
365 pathogenic inflammation we have characterised the local
366 response to mammary inflammation in mice, and have
367 demonstrated increased expression of various inflammatory
368 cytokines and APPs. We have shown that the response to
369 pathological inflammation (infection with *Staph. aureus*) is
370 different from the response caused by 'physiological inflam-
371 mation' (due to milk accumulation). The expression of
372 inflammatory cytokines was increased only in individual
373 glands inoculated with the pathogen, whereas APP ex-
374 pression was increased in both pathogen-inoculated and
375 teat-sealed glands. The time course of the APP response was
376 similar for the two inflammatory states, with the exception
377 that SAA3 remained elevated beyond 48 h in pathologically
378 inflamed glands, but not in physiologically inflamed.

379 Milk contains a great many bioactive factors, but the
380 mammary gland is a complex structure, hence in many cases
381 the precise origin of these factors (including cytokines and
382 APPs) has not been totally elucidated. Proteolytic
383 breakdown of synthesised protein (largely caseins) during
384 storage, transcellular trafficking and secretion of intact
385 molecules from plasma to milk, paracellular transfer of mol-
386 ecules through 'leaky' tight junctions and de-novo synthesis
387 and secretion by mammary epithelial cells are all possible
388 sources of proteins and peptides in milk. It must also be
389 recognised that detection of mammary expression of a
390 particular protein does not necessarily imply production by
391 mammary secretory cells, since the tissue is very highly
392 vascularised. Where cytokines are concerned, it is important
393 to recognise that mastitic milk contains very large numbers
394 of neutrophils and other immune cells, and whilst mammary
395 epithelial cells have been shown to synthesise certain
396 cytokines (Goldman et al. 1996) it is likely that the majority
397 of cytokines in mastitic milk will be derived from immune
398 cells of one sort or another. The situation is less clear for
399 APPs. On the basis of milk/plasma profile comparison and
400 expression within mammary tissue, Eckersall et al. (2006)
401 proposed that MSAA3 was a specific product of mammary
402 tissue, and our data support that conclusion. Pedersen et al.
403 (2003) used a bovine *Streptococcus uberis* model and
404 observed a marked increase in milk HP without a con-
405 comitant increase in plasma levels, and Hiss et al. (2004)
406 observed expression of HP in mammary tissue after
407 endotoxin challenge. Nevertheless, Lai et al. (2009) consider
408 that neutrophils are the major source of HP in mastitic milk,
409 although they also showed epithelial expression. Expression
410 of AGP in mammary tissue has been shown (Ceciliani et al.
411 2005, 2007), but the same group subsequently demonstrated
412 that bovine neutrophils also express AGP (Rahman et al.
413 2008). To our knowledge, AGP has not previously been
414 investigated in relation to mastitis. Our expression data
415 provide additional indirect evidence for production of all
416 three APPs by mastitic mammary tissue, as opposed to im-
417 mune cells contained within the sample. Although we did
418 not measure immune cells in our milk (or tissue) samples, the
419 fact that cytokine levels increased in the *Staph. aureus*-
420 challenged glands but not appreciably in the teat-sealed
421 glands leads us to suggest that the number of immune cells
422 was probably greater in the former than the latter. In support
423 of this, Brouillette et al. (2004) observed marked infiltration

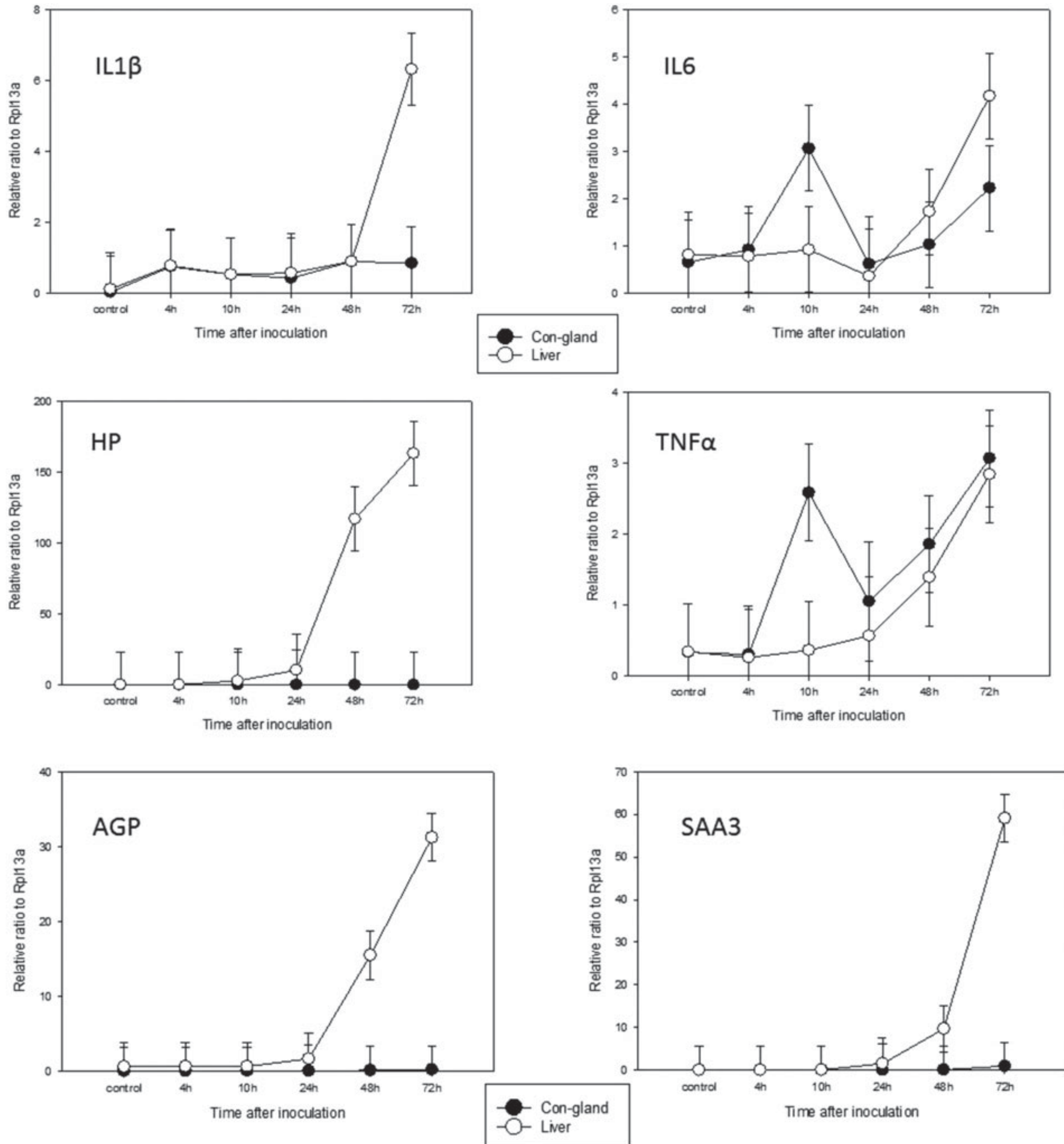

Fig. 2. Relative expression of IL1β, IL6, TNFα, HP, AGP and SAA3 ± SE between control glands and liver samples within time intervals.

of polymorphonuclear monocytes 12 h after inoculation with *Staph. aureus* in a mouse mastitis model. Despite this likely difference in immune cells, the expression of HP and AGP was identical in the two scenarios, and the expression of SAA3 was very similar prior to 72 h. This time-point has physiological relevance for the mammary involutionary process in mice. Sorensen & Knight (1997, unpublished data) showed that murine lactation could be 'rescued' by resuckling after periods of non-suckling of up to 72 h, but not thereafter. Stein et al. (2004) demonstrated a biphasic immune response in involuting mouse mammary tissue, with an initial and rapid acute phase response followed by significant infiltration of neutrophils and macrophages only after day 4, providing additional confirmation that our data are most likely to represent actual mammary expression. At the same time, it is important to recognise that our current model represents involution due entirely to local factors within the gland, since the systemic suckling stimulus was maintained throughout. From a diagnostic point of view our model is appropriate; detecting non-pathogenic mastitis due

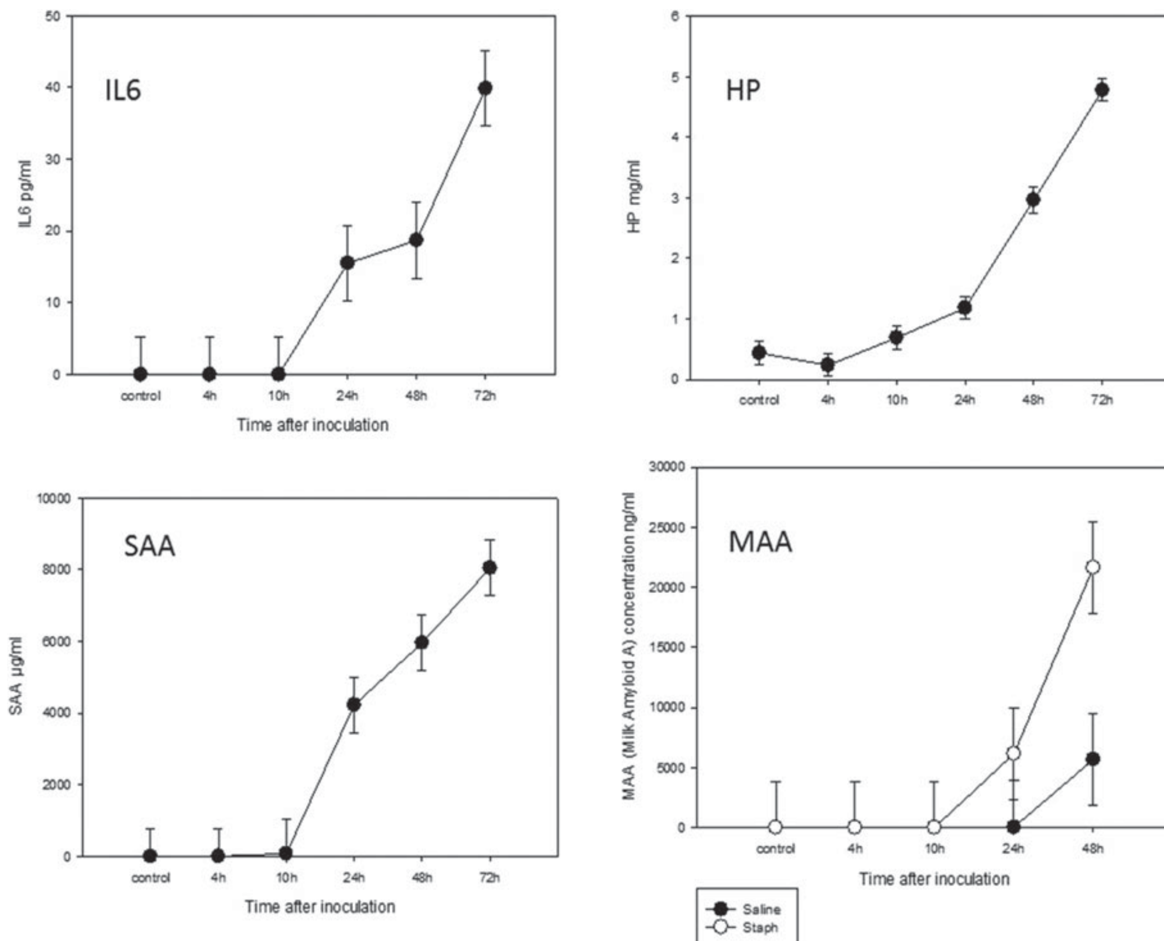

Fig. 3. Concentration of SAA, HP and IL6 in blood serum ± SE between groups. MAA concentration ± SE between control group, saline and staph injected groups within time intervals.

to milk accumulation in the context of bovine or human lactation would be done when milking or suckling was continuing. On the basis of our data, we can propose that the combination of cytokine and APP measurement might allow for discrimination between pathogenic and non-pathogenic mastitis. Such discrimination is important, since the former would be likely to respond to antibiotic treatment whereas the latter would not.

The normal course of events in an inflammatory response is for local signals originating at the site of inflammation to subsequently activate hepatic cytokine and APP production (Yoshioka et al. 2002). We observed increased mammary expression of IL6 at 10 h, but the significance of this observation is uncertain because it was only in the control gland. Plasma levels of IL6 increased at 24 h, but hepatic expression remained low until 48 h. There was a generalised increase of mammary TNFα expression at 10 h, but overall our data do not shed significant light on the local signals that provide the earliest indication of an infection. IL1β had the highest and also earliest mammary expression response to *Staph. aureus* and is probably worthy of more detailed study if an early diagnostic tool is the target. Gunther et al. (2011) compared *Escherichia coli* and *Staph. aureus* challenge of bovine MEC in vitro and observed very marked and early stimulation of TNFα, IL6 and IL1A in response to the former, but not the latter. It is likely that early detection will be particularly challenging where *Staph. aureus* mastitis is concerned. Similar conclusions have been drawn by others (Bannerman et al. 2004; Petzl et al. 2008). The plasma cytokine analysis that we performed should have been capable of detecting additional cytokines had they been present, which may have been a methodological problem but could also have indicated low responsiveness to *Staph. aureus*. The large increase that we observed in plasma IL1β at 72 h was accompanied by symptoms of systemic infection in some of the mice, including lack of normal behaviour, grooming and loss of appetite. Interpretation of our mammary data must take these systemic responses into account. In quantitative terms, we observed much greater expression of APP by liver than by mammary gland, but the increased plasma levels of APP were first observed at 24 h, in advance of both hepatic activation and the major increases in mammary expression. The one APP that we measured in milk, MAA was at much lower concentration than SAA in blood, but these data are

not directly comparable since they were obtained using different antibodies. Kovacevic-Filipovic et al. (2012) have recently compared bovine milk and plasma SAA and observed rather similar concentrations during *Staph. aureus* mastitis, but on the basis of isoform distribution concluded that plasma SAA and M-SAA did not simply equilibrate through leaky tight junctions. Our data do not allow us to speculate on the relative importance of locally or systemically produced APP in local host defence within the mammary gland. Given the different degrees of up-regulation, it is possible that liver is the major site of APP production, acting in response to inflammatory cytokines produced by the mammary gland. However, this cannot be stated with certainty.

In conclusion, we observed different patterns of mammary expression of inflammatory cytokines and APPs in pathogenic *vs.* non-pathogenic mammary inflammation in a mouse model. This could potentially form the basis for differential diagnosis of the two conditions. Further research is needed to clarify the interrelationships between cytokine and APP production in mammary tissue and liver.

References

Akerstedt M, Forsback L, Larsen L & Svennersten-Sjaunja K 2011 Natural variation in biomarkers indicating mastitis in healthy cows. *Journal of Dairy Research* **78** 88–96

Anderson JC 1977 Experimental staphylococcal mastitis in the mouse: the induction of chronic mastitis and its response to antibiotic therapy. *Journal of Comparative Pathology* **87** 611–621

Bannerman DD 2009 Pathogen-dependent introduction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. *Journal of Animal Science* **87** 10–25

Bannerman DD, Paape MJ, Lee J, Zhao X, Hope JC & Rainard P 2004 *Escherichia coli* and *staphylococcus aureus* elicit differential innate immune response following intramammary infection. *Clinical and Diagnostic Laboratory Immunology* **11** 463–472

Bayles KW, Wesson CA, Liou LE, Fox LK, Bohanch GA & Trumble WR 1998 Intracellular *Staphylococcus aureus* escape the endosome and induces apoptosis in epithelial cells. *Infection and Immunity* **66** 336–342

Brouillette E & Malouin F 2005a The pathogenesis and control of *Staphylococcus aureus*-induced mastitis: study models in the mouse. *Microbes and Infection* **7** 560–568

Brouillette E, Grondin G, Lefebvre C, Talbot BG & Malouin F 2004 Mouse mastitis model of infection for antimicrobial compound efficacy studies against intracellular and extracellular forms of *Staphylococcus aureus*. *Veterinary Microbiology* **101** 253–262

Brouillette E, Grondin G, Talbot BG & Malouin F 2005b Inflammatory cell infiltration as an indicator of *Staphylococcus aureus* infection and therapeutic efficacy in experimental mouse mastitis. *Veterinary Immunology and Immupathology* **104** 163–169

Ceciliani F, Pocacqua V, Provasi E, Comunian C, Bertolini A, Bronzo V, Moroni P & Sartorelli P 2005 Identification of the bovine α1-acid glycoprotein in colostrum and milk. *Veterinary Research* **36** 735–746

Ceciliani F, Pocacqua V, Lecchi C, Fortin R, Rebucci R, Avallone G, Bronzo V, Cheli F & Sartorelli P 2007 Differential expression and secretion of a1-acid glycoprotein in bovine milk. *Journal of Dairy Research* **74** 374–380

Cha E, Bar D, Hertl JA, Tauer LW, Bennett G, Gonzalez RN, Schukken YH, Welcome FL & Grohn YT 2011 The cost and management of different types of clinical mastitis in dairy cows by dynamic programming. *Journal of Dairy Science* **94** 4476–4487

Chandler RL 1970 Experimental bacterial mastitis in the mouse. *Journal of Medical Microbiology* **3** 273–289

Eckersall PD 1995 Acute phase proteins as markers for inflammatory lesions. *Comparative Haemotology International* **5** 93–97

Eckersall PD & Bell R 2010 Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. *Veterinary Journal* **185** 23–27

Eckersall PD, Young FJ, Nolan AM, Knight CH, McComb C, Waterson MM, Hogarth CJ, Scott EM & Fitzpatrick JL 2006 Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus* subclinical mastitis. *Journal of Dairy Science* **89** 1488–1501

Eckersall PD, Lawson FP, Bence L, Waterson MM, Lang TL, Donachie W & Fontains MC 2007 Acute phase protein response in an experimental model of ovine caseous lymphadenitis. *BMC Veterinary Research* **3** 35

Goldman AS, Chheda S, Garofalo R & Schmalstieg C 1996 Cytokines in human milk: properties and potential effects upon the mammary gland and the neonate. *Journal of Mammary Gland Biology and Neoplasia* **1** 251–258

Gunther J, Katrin E, Poschadel N, Petzl W, Holm Z, Simone M, Helmut B & Hans-Martin S 2011 Comparative kinetics of *Escherichia coli- and Staphylococcus aureus*-specific activation of key-immune pathways in mammary epithelial cell demonstrates that *S. aureus* elicits a delayed response dominated by Interleukin-6 (IL-6) but not by IL-1A or Tumor necrosis factor alpha. *Infection and Immunity* **79** 695–707

Han LQ, Yang GY, Zhu HS, Wang YY, Wang LF, Guo YJ, Lu WF, Li HJ & Wang YL 2010 Selection and use of reference genes in mouse mammary glands. *Genetics and Molecular Research* **9** 449–456

Hensen SM, Pavicic MJ, Lohuis JA, de Hoog JA & Poutrel B 2000 Location of *Staphylococcus aureus* within experimentally infected bovine udder and expression of capsular polysaccharide type 5 in situ. *Journal of Dairy Science* **83** 1966–1975

Hiss S, Mielenz M, Bruckmaier RM & Sauerwein H 2004 Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. *Journal of Dairy Science* **87** 3778–3784

Hu C, Gong R, Guo A & Chen H 2010 Protective effect of ligand-binding domain of fibronectin-binding protein on mastitis induced by *Staphylococcus aureus* in mice. *Vaccine* **28** 4038–4044

Koj A 1996 Initiation of acute phase response and synthesis of cytokines. *Biochimica et Biophysica Acta* **1317** 84–94

Kovacevic-Filipovic M, Ilic V, Vujcic Z, Dojnov B, Stevanov-Pavlovic M, Mijacevic Z & Bozic T 2012 Serum amyloid A isoforms in serum and milk from cows with *Staphylococcus aureus* subclinical mastitis. *Veterinary Immunology and Immunopathology* **145** 120–128

Kvist LJ 2013 Re-examination of old truths: replication of a study to measure the incidence of lactational mastitis in breastfeeding women. *International Breastfeeding Journal* **8**

Lakic B, Sjaunja KS, Norell L, Dernfalk J & Ostensson K 2011 The effect of a single prolonged milking interval on inflammatory parameters, milk composition and yield in dairy cows. *Veterinary Immunology and Immunopathology* **140** 110–118

Lai IH, Tsao JH, Lu YP, Lee JW, Zhao X, Chien FL & Mao SJ 2009 Neutrophils as one of the major haptoglobin sources in mastitis affected milk. *Veterinary Research* **40** 17

McDonald TL, Larson MA, Mack DR & Weber A 2001 Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum. *Veterinary Immunology and Immunopathology* **83** 203–211

Murata H, Shimada N & Yoshioka M 2004 Current research on acute phase proteins in veterinary diagnosis: an overview. *Veterinary Journal* **168** 28–40

Nielsen BH, Jacobsen S, Andersen PH, Niewold TA & Heegaard PMH 2004 Acute phase protein concentration in serum and milk from healthy cows, cows with clinical mastitis and cows with extramammary inflammatory conditions. *Veterinary Record* **154** 361–365

Pedersen LH, Aalbæk B, Røntved CM, Ingvartsen KL, Sorensen NS, Heegaard PM & Jensen HE 2003 Early pathogenesis of inflammatory response in experimental bovine mastitis due to *Streptococcus uberis*. *Journal of Comparative Pathology* **128** 156–164

615 **Peli A, Scagliarini A, Britti D & Boari A** 2003 Detection of proinflammatory
616 and regulatory cytokines in bovine milk using RT-PCR. *Veterinary*
617 *Research Communication* **27** 779–781
618 **Petzl W, Zerbe H, Gunther J, Yang W, Seyfert H, Nurnberg G &**
619 **Schuberth H** 2008 *Escherichia coli*, but not *Staphylococcus aureus*
620 triggers an early increased expression of factors contributing to the innate
621 immune defense in the udder of the cow. *Veterinary Research* **39** 18
622 **Pfaffl MW** 2001 A new mathematical model for relative quantification in
623 real-time RT-PCR. *Nucleic Acids Research* **29** e45
624 **Quarrie LH, Addey CV & Wilde CJ** 1996 Programmed cell death during
625 mammary tissue involution induced by weaning, litter removal, and milk
626 stasis. *Journal of Cellular Physiology* **168** 559–569
627 **Rahbek M, Nazemi S, Ødum L, Gupta S, Poulsen SS, Hay-Schidt A &**
628 **Klærke DA** 2014 Expression of the small conductance Ca^{2+}-activated
629 potassium channel subtype 3 (SK3) in rat uterus after stimulation with
630 17-β-estradiol. *PLoS ONE* **9** e87652
631 **Rahman MM, Miranda-Ribera A, Lecchi C, Bronzo V, Sartorelli P,**
632 **Franciosi F & Ceciliani F** 2008 Alpha1-acid glycoprotein is contained
633 in bovine neutrophil granules and released after activation. *Veterinary*
634 *Immunology and Immunopathology* **125** 71–81
635 **Roberson JR** 2012 Treatment of clinical mastitis. *Veterinary Clinics of*
636 *North America: Food Animal Practice* **28** 271–288
637 **Schukken YH, Gunther J, Fitzpatrick J, Fontaine MC, Goetze L, Holst O,**
638 **Leigh J, Petzl W, Schuberh HJ, Sipka A, Smith DGE, Quesnell R, Watts J,**
639 **Yancey R, Zerbe H, Gurjar A, Zadoks RN & Seyfert HM**; members of the
640 Pfizer mastitis research consortium 2011 Host-response patterns of
641 intramammary infection in dairy cows. *Veterinary Immunology and*
642 *Immunopathology* **144** 270–289
643 **Scott JA, Robertson M, Fitzpatrick J, Knight C & Mulholland S** 2008
644 Occurrence of lactational mastitis and medical management: a
prospective cohort study in Glasgow. *International Breastfeeding* 645
Journal **3** 21 646
Seegers H, Fourichon C & Beaudeau F 2003 Production effects related to 647
mastitis and mastitis economics in dairy cattle herds. *Veterinary Research* 648
34 475–491 649
Shipman LJ, Docherty AH, Knight CH & Wilde CJ 1987 Metabolic 650
adaptations in mouse mammary gland during a normal lactation cycle 651
and in extended lactation. *Quarterly Journal of Experimental Physiology* 652
72 303–311 653
Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy M, Heath VJ, Bell AK, 654
Ferrier RK, Sandilands GP & Gusterson BA 2004 Involution of the mouse 655
mammary gland is associated with an immune cascade and an acute- 656
phase response, involving LBP, CD14 and STAT3. *Breast Cancer* 657
Research **6** 75–91 658
Theil PK, Labouriau R, Sejrsen K, Thomsen B & Sørensen MT 2005 659
Expression of gene involved in regulation of cell turnover during milk 660
stasis and lactation rescue in sow mammary glands. *Journal of Animal* 661
Science **83** 2349–2356 662
Villalba M, Hott M, Martin C, Aguila B, Valdiva S, Quezada C, Zambrano A, 663
Concha MI & Otth C 2012 Herpes simplex virus type 1 induces 664
simultaneous activation of toll-like receptors 2 and 4 and expression of 665
the endogenous ligand serum amyloid A in astrocytes. *Medical* 666
Microbiology and Immunology **201** 371–379 667
Wang S, Diao H, Guan Q, Cruikshank WW, Delovitch TL, Jevnikar AM 668
& Du C 2008 Decreased renal ischemia-reperfusion injury by IL-16 669
inactivation. *Kidney International* **73** 318–326 670
Yoshioka M, Watanabe A, Shimada N, Murata H, Yokomizo Y & Nakajima Y 671
2002 Regulation of haptoglobin secretion by recombinant bovine 672
cytokines in primary cultured bovine hepatocytes. *Domestic Animal* 673
Endocrinology **23** 425–433 674