Technical note: A pilot study using a mouse mastitis model to study differences between bovine associated coagulase-negative staphylococci

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ABSTRACT

Coagulase-negative staphylococci (CNS) are a group of bacteria classified as either minor mastitis pathogens or commensal microbiota. Recent research suggests species- and even strain-related epidemiological and genetic differences within the large CNS group. The current pilot study investigated in 2 experiments whether a mouse mastitis model validated for bovine *Staphylococcus aureus* can be used to explore further differences between CNS species and strains. In a first dose titration experiment, a low inoculum dose of *S. aureus* Newbould 305 (positive control) was compared with increasing inoculum doses of a *Staphylococcus chromogenes* strain originating from a chronic bovine intramammary infection to a sham-inoculated mammary glands (negative control). In contrast to the high bacterial growth following inoculation with *S. aureus*, *S. chromogenes* was retrieved in very low levels at 24 h postinduction (p.i.). In a second experiment, the inflammation inflicted by 3 CNS strains was studied in mice. The host immune response induced by the *S. chromogenes* intramammary strain was compared with the one induced by a *Staphylococcus fleurettii* strain originating from cow bedding sawdust and by a *S. chromogenes* strain originating from a teat apex of a heifer. As expected, at 28 and 48 h p.i., low bacterial growth and local neutrophil influx in the mammary gland were induced by all CNS strains. As hypothesized, bacterial growth p.i. was the lowest for *S. fleurettii* compared with that induced by the 2 *S. chromogenes* strains, and the overall immune response established by the 3 CNS strains was less pronounced compared with the one induced by *S. aureus*. Proinflammatory cytokine profiling revealed that *S. aureus* locally induced IL-6 and IL-1β but not TNF-α, whereas, overall, CNS-inoculated glands lacked a strong cytokine host response but also induced IL-1β locally. Compared with both other CNS strains, *S. chromogenes* from the teat apex inflicted a more variable IL-1β response characterized by a more intense local reaction in several mice. This pilot study suggests that an intraductal mouse model can mimic bovine CNS mastitis and has potential as a complementary in vivo tool for future CNS mastitis research. Furthermore, it indicates that epidemiologically different bovine CNS species or strains induce a differential host innate immune response in the murine mammary gland.
Key words: coagulase-negative staphylococci strain, mouse mastitis model, bacterial growth, neutrophil influx, proinflammatory cytokine

Technical Note

Coagulase-negative staphylococci are a group of bacteria that cause mastitis in both heifers and multiparous cows (Thorberg et al., 2009; De Vliegher et al., 2012; Ajitkumar et al., 2013). Until recently, it was difficult to draw consistent conclusions on the relevance of CNS for bovine udder health. Some studies considered CNS as true mastitis pathogens, although most were retrieved from subclinical mastitis cases (Pyörälä and Taponen, 2009), whereas others considered CNS to be commensal bacteria with limited or absent negative effects on SCC, milk quality, and milk production (Schukken et al., 2009; De Vliegher et al., 2012). The development and validation of molecular identification techniques specifically for bovine-associated CNS were the basis to eliminate this confusion (Zadoks and Watts, 2009). Whereas older studies dealt with CNS as one homogenous and coherent group (Schukken et al., 2009; Piepers et al., 2010; Piepers et al., 2013), more recent studies addressed the CNS species separately, revealing heterogeneous characteristics between them (Avall-Jääskeläinen et al., 2013). In essence, 12 CNS species are frequently isolated from bovine milk. Evidence exists for diversity between and even within those species in their epidemiologic behavior and traits, such as persistence, antibiotic resistance, susceptibility to phagocytosis by mouse macrophages, and (putative) virulence (Piessens et al., 2011; Supré et al., 2011;

Avall-Jääskeläinen et al., 2013; Vanderhaeghen et al., 2014).

To make further progress in our understanding of CNS mastitis, the interaction between CNS species and strains and the host should be studied in more detail. Ideally, such experimental infection studies are conducted using dairy cows. Unfortunately, these bovine studies are expensive and labor intensive. For those reasons, a mouse mastitis model was developed and characterized (Chandler, 1970) and has since been successfully validated for studying the specific host immune response to IMI with major mastitis pathogens, such as *Escherichia coli* and *Staphylococcus aureus* (Brouillette and Malouin, 2005; Demon et al., 2012; Demon et al., 2013), but not for CNS.

The usefulness of a mouse mastitis model as a complementary tool to investigate differences between bovine CNS species and strains was explored. Specifically, 2 experiments were performed to answer 3 clear-cut objectives: (1) whether bovine-associated CNS grow in the murine mammary gland, (2) whether they induce mastitis in mice, and (3) whether it is possible to detect differences in bacterial growth, clinical symptoms, and host immune response between CNS species or strains with a different origin using this mouse mastitis model.

The set-up of the 2 experiments is outlined in Figure 1. For both experiments, 8-wk-old female Hsd:ICR (CD1) mice that mated with 10-wk-old male Hsd:ICR (CD1) mice (Harlan Laboratories, Horst, the Netherlands) were used. Pups were weaned ±10 d after parturition to ease the intraductal accessibility. All inoculations were performed 2 h postweaning under isoflurane anesthesia combined with a long-acting analgesic buprenorphine (10 μg/kg of Vetergesic, Patheon UK Ltd., Swindon, UK) using a 32-gauge blunt needle. During both experiments, core body temperature of the mice was measured with a rectal thermistor. All experiments were approved by the committee on the ethics of animal experiments of Faculty of Veterinary Medicine, University of Ghent (permit number: EC2013/166).

In a first dose titration experiment, 4 increasing inoculum doses (i.e., 2.5×10^2, 2.5×10^3, 2.5×10^4, and 2.5×10^5 cfu/100 μL) of a bovine-associated *Staphylococcus chromogenes* strain originating from a chronic IMI [**IM** (Supré et al., 2011)] were injected in the fourth gland pair of each of the 12 mice ($n_{mice/dose} = 3$; $n_{glands/dose} = 6$) to evaluate both bacterial growth and immune cell influx in the mammary glands 24 h postinduction (**p.i.**). An additional 6 mice were inoculated with either a 100-μL sham solution (PBS + 10% glycerol; $n_{mice} = 3$) or 1×10^2 cfu/100 μL of *Staphylococcus aureus* Newbould 305 ($n_{mice} = 3$) and were included as negative and positive controls, respectively (Figure 1A). Twenty-four hours p.i., all mice were first sedated by administer-

ing a mixture of ketamine (100 mg/kg of Anesketin, Eurovet Animal Health BV, Bladel, the Netherlands) with xylazine (10 mg/kg; Xylazini Hydrochloridum, Val d'Hony-Verdifarm, Beringen, Belgium) intraperitoneally and subsequently euthanized. After euthanasia, all *S. chromogenes*-inoculated glands ($n_{glands/inoculum} = 6$) were isolated to determine their bacterial load. Likewise, the *S. chromogenes*-inoculated glands with clinical signs of inflammation after dissection (i.e., red color, hard, swollen), as well as the 2 sham- and 2 *S. aureus*-inoculated glands, were further processed for histology. To quantify bacterial growth, the mammary glands were weighed, homogenized, and spotted (20 μL) in serial logarithmic dilutions on Tryptic soy agar plates (Oxoid, Drongen, Belgium) overnight at 37°C to determine colony-forming units per 100 μL and divided by the exact weight of a mammary gland (g). For the histologic sections, mammary glands were divided in 3 transverse proportions to validate the homogeneity of the infection: near the nipple (i.e., near the inoculation site), near the lymph node, and near the back. Two mammary glands for every inoculation (sham-, *S. aureus*-, and 10^5 cfu of *S. chromogenes* IM-inoculated glands) were fixed in buffered 3.5% formaldehyde (Sigma-Aldrich, St. Louis, MO) for 24 h at room temperature (24°C). Subsequently, the samples were dehydrated and embedded in paraffin wax. Sections were deparaffinized, hydrated, and stained with hematoxylin and eosin (Sigma-Aldrich). As the inoculated mammary glands displayed clinical symptoms of inflammation following a *S. chromogenes* IM inoculum dose as high as 10^5 cfu, but not at lower doses, only these CNS-inoculated glands were collected for histological evaluation.

In the second experiment, 2 additional bovine CNS strains were included [a *Staphylococcus fleurettii* strain originating from sawdust (Supré et al., 2011) and a *S. chromogenes* strain originating from a teat apex (**TA**) of a heifer (De Vliegher et al., 2004)]. The fourth gland pair of each of the 18 mice ($n_{mice/CNS strain} = 6$; $n_{glands/CNS strain} = 12$) were injected with either *S. chromogenes* IM, *S. chromogenes* TA, or *S. fleurettii* at an inoculum dose of approx. 10^5 cfu (9.6×10^4 cfu/100 μL of *S. chromogenes* IM, 9.3×10^4 cfu/100 μL of *S. chromogenes* TA, and 8.5×10^4 cfu/100 μL of *S. fleurettii*, respectively). Three additional mice were inoculated with 2.5×10^2 cfu/100 μL of *S. aureus* Newbould 305 ($n_{mice/dose} = 3$; $n_{glands/dose} = 6$) and included as a positive control (Figure 1B). The fifth gland pair of each mouse was inoculated with 100 μL of sham solution and served as negative control. At 28 h p.i., 12 mice (3 mice per CNS strain and 3 *S. aureus* Newbould 305-inoculated mice) were sedated and euthanized as previously described. The other mice (n = 9) were se-
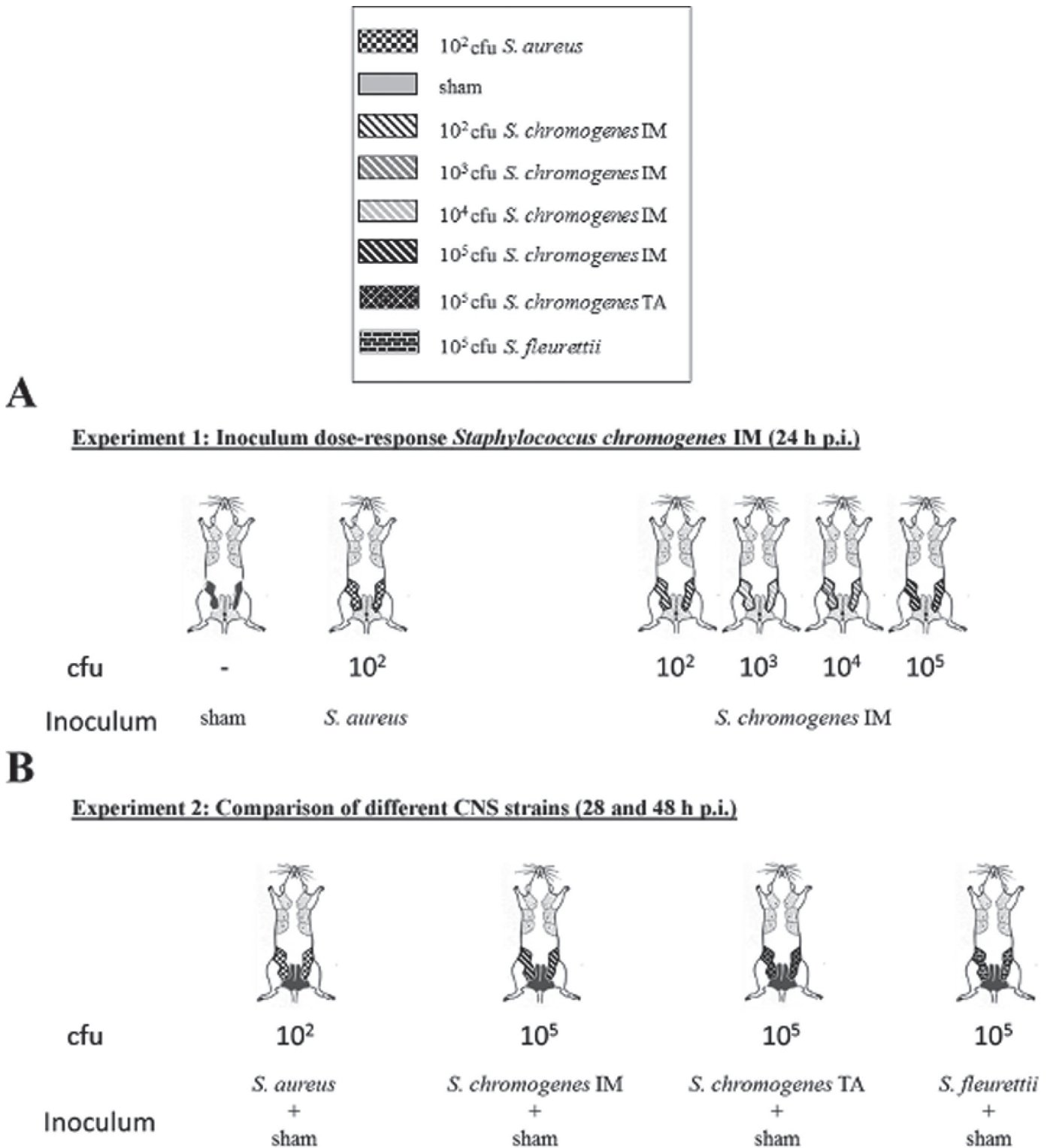
Figure 1. Schematic presentation of the set-up of the 2 experiments (A and B) performed in the current study. The patterned glands represent the glands inoculated with *Staphylococcus aureus*, *Staphylococcus chromogenes* intramammary (IM), *S. chromogenes* teat apex (TA), or *Staphylococcus fleurettii*, whereas the gray and dark gray mammary glands represent the unchallenged and the PBS/glycerol (sham)-inoculated mammary glands, respectively. p.i. = postinfection.

dated and euthanized at 48 h p.i. After euthanasia, the inoculated glands were isolated to determine bacterial growth, cytokine levels, and histology. Bacterial growth was determined for every inoculum and time point as described. For cytokine quantification, mammary gland lysates were prepared from the mammary homogenates as previously reported (Breyne et al., 2014). Briefly, the IL-6, IL-1β levels, and TNF-α were determined in the protein suspensions (50 μg) as described by the manufacturer with specific cytometric Bead Array kits (CBA, Becton Dickinson Biosciences, Erembodegem, Belgium) on a FACSArray instrument (Becton Dickinson Biosciences). For histology, a sagittal section was used. Two mammary glands for every inoculum and time point were fixed and prepared as described. Statistical differences in bacterial growth at 28 and 48 h p.i. and between the 3 CNS strains were determined using nonparametric or ANOVA tests (SPSS 20.0, IBM Corporation, Armonk, NY) with the $\log_{10}$-transformed colony-forming units per milliliter as outcome variables and the 3 CNS strains as the categorical independent variable. A similar approach was followed to determine the association between the inoculum (*S. aureus*, *S. chromogenes* IM, *S. chromogenes* TA, or *S. fleurettii*) and the IL-6, IL-1β, and TNF-α concentration either 28 or 48h p.i. For parametric testing, mean values of inoculation groups were compared through Tukey post hoc testing. A *P*-value lower than 0.05 was considered statistically significant, values are represented as mean with SD.

Bacteria were extracted as described previously from randomly chosen CNS-inoculated mammary glands ($n_{glands/CNS\ strain}$ = 4) to validate the results of both experiments; commensal microbiota was determined in unchallenged lactating glands and sham-inoculated mammary glands (n = 9 and n = 4). All colonies were counted on Tryptic soy agar plates and streaked onto Columbia agar (Oxoid) to obtain pure cultures. After 24 h of aerobic incubation at 37°C, colonies were checked for purity and identified following National Mastitis Council procedures (Hogan et al., 1999). All CNS were identified to the species level using transfer RNA-intergenic spacer PCR (Supré et al., 2009). If no identification could be obtained, isolates were subjected to sequencing of the 16S rRNA gene (Park et al., 2011).

Experiment 1: IMI of Mice with Increasing Inoculum Doses of S. chromogenes IM

Staphylococcus chromogenes IM-inoculated glands at 24 h p.i. displayed higher bacterial levels compared with sham-inoculated glands. Still, bacterial growth in the *S. chromogenes* IM inoculated glands was limited compared with the *S. aureus* Newbould 305-inoculated
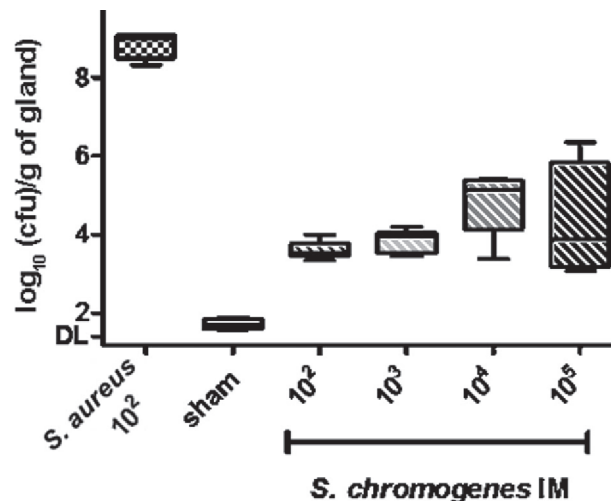

Figure 2. Bacterial growth (expressed as $\log_{10}$ cfu/g of gland) in mammary glands of mice inoculated at 24 h postinfection (p.i.) with PBS/glycerol (sham), *Staphylococcus aureus*, and *Staphylococcus chromogenes* intramammary (IM) in experiment 1. The line in each box represents the median of these individual data sets. The color and pattern code of each group correspond to these explained in Figure 1. Error bars represent the standard deviation. DL = detection limit.

glands, regardless of the inoculum dose (Figure 2 and Table 1). The presence of *S. chromogenes* in the challenged glands was confirmed. A marked increase in mammary colony-forming units p.i. was observed in *S. aureus* Newbould 305-inoculated glands compared with both the sham- as well as *S. chromogenes* IM-inoculated glands (Figure 2).

Upon histological evaluation, both *S. aureus* and *S. chromogenes* IM induced an influx of immune cells in the alveoli (Figure 3B and C) that was absent in sham-inoculated glands (Figure 3A). The latter had an intact alveolar structure with a swollen luminal area at 24 h p.i. with accumulated milk. In *S. aureus*-inoculated mammary glands, the general structural integrity of the alveoli was still present but the milk was replaced by a vast number of invading cells (Figure 3B; white arrow heads) that were tentatively identified as neutrophils mainly based on their characteristic polymorphonuclear feature. In analog, the alveoli from the *S. chromogenes* IM-inoculated glands were also packed with immune cells (Figure 3C; white arrow heads). This influx of cells in the mouse mammary gland was more invasive following *S. aureus* inoculation, as red blood cells were clearly observed in the interstitium, which was not the case after inoculation with *S. chromogenes* IM (Figure 3B; black arrow heads).

Experiment 2: IMI of Mice with Different CNS Strains

None of the 3 tested CNS strains had a significant overall change in the number of colony-forming units

Table 1. Overview of the amount of bacteria ($\log_{10}$cfu/g) in mammary glands at 24 h post infection (p.i.) of mice inoculated with *Staphylococcus aureus*, PBS/glycerol (sham), and *Staphylococcus chromogenes* IM of experiment 1

Item	S. aureus (10^2)	Sham	S. chromogenes IM			
			10^2	10^3	10^4	10^5
Minimum	8.32	1.57	3.34	3.44	3.38	3.08
25th Percentile	8.49	1.60	3.47	3.51	4.14	3.18
Median	9.00	1.72	3.54	3.95	5.13	3.87
75th Percentile	9.06	1.86	3.76	4.04	5.38	5.84
Maximum	9.08	1.89	3.99	4.19	5.41	6.37

between 28 and 48 h p.i. At both time points, no significant difference in bacterial growth was detected between *S. chromogenes* IM (6.94 ± 1.56 $\log_{10}$ cfu/g of gland) and *S. chromogenes* TA (7.13 ± 2.11 $\log_{10}$ cfu/g of gland; Figure 4). In contrast, *S. fleurettii* (4.41 ± 1.01 $\log_{10}$ cfu/g gland) was retrieved at significantly lower levels compared with both *S. chromogenes* strains ($P < 0.01$). Moreover, the first was not grown in the murine mammary gland but seems even decreased in

$\log_{10}$ colony-forming units per gram of gland p.i. at 28 or 48 h compared with the administered inoculum. In analog with the first experiment, *S. aureus* displayed an exponential bacterial growth compared with each of the CNS p.i. The presence of each inoculated strain p.i. was confirmed. No distinct differences in histological features were observed between both time points p.i. All tested strains induced an influx of immune cells, of which the majority likely consisted of neutrophils. Still,
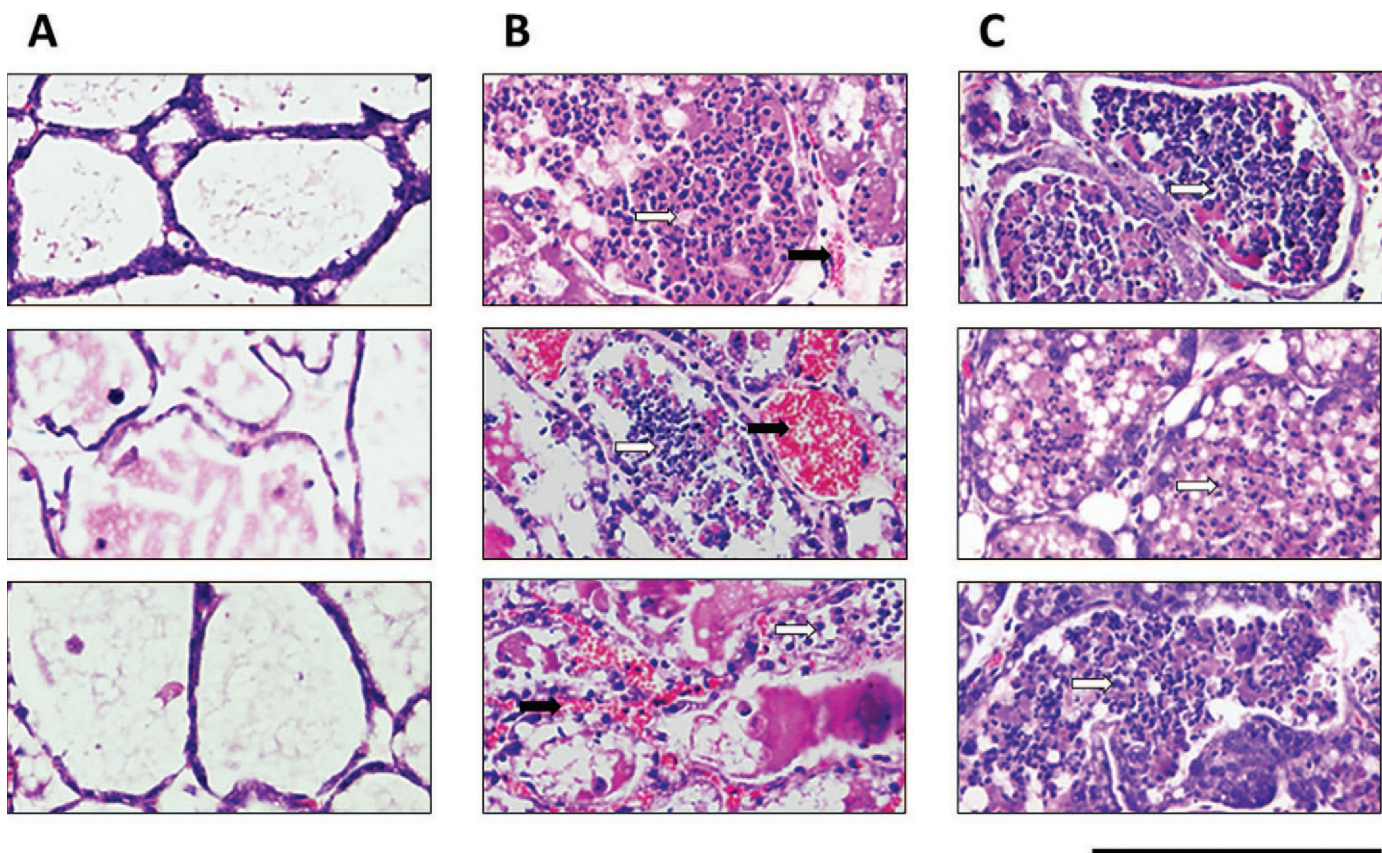

Figure 3. Representative hematoxylin and eosin-stained sections of mammary glands isolated from mice inoculated with (A) PBS/glycerol (sham), (B) *Staphylococcus aureus*, and (C) *Staphylococcus chromogenes* in experiment 1. The histology images in the upper panel are taken from sections sampled near the nipple, the ones in the middle panel near the subiliac lymph node, and the ones in the lowest panel near the dorsal gland tissue. The white arrows mark the immune cell content in the alveoli, whereas the black arrows point to the red blood cells in the interstitium. The scale bar represents a length of 200 µm. Color version available online.

in marked contrast to *S. aureus*, no vascular congestion was observed in the interstitium after challenge with each of the CNS strains (Figure 5A, B, C, and D). Corroborating the results of the first experiment, sham-inoculated control gland alveoli had an enlarged lumen filled with milk and lacked the presence of immune cells (Figure 5E). Both *S. chromogenes* strains had a similar scattered distribution of immune cells in contrast with the densely packed neutrophils retrieved in the alveolar lumen of the mouse mammary glands upon inoculation with *S. fleurettii* (Figure 5A, B, and C). Although a lower inoculum dose was injected, cytokine quantification revealed significantly higher IL-6 levels in the *S. aureus*-inoculated glands ($3.11 \pm 0.17 \log_{10}$ IL-6 pg/mL) than in the sham- ($0.93 \pm 0.19 \log_{10}$ IL-6 pg/mL) or CNS-inoculated glands ($1.15 \pm 0.56 \log_{10}$ IL-6 pg/mL; $P < 0.001$; Figure 6A). Furthermore, significant differences in the local IL-6 concentrations were not observed between the 3 CNS strains, between the 2 time points p.i. within strains, or between the sham- and CNS-inoculated glands. A similar trend was observed for the local IL-1β levels, which were substantially higher in *S. aureus*-inoculated glands ($2.97 \pm 0.14 \log_{10}$ IL-1β pg/mL), compared with the sham- ($1.06 \pm 0.42 \log_{10}$ IL-1β pg/mL, $P < 0.001$) and CNS-inoculated glands ($1.87 \pm 0.61 \log_{10}$ IL-1β pg/mL; $P < 0.05$; Figure 6B). Still, the differences in local IL-1β concentrations induced by *S. aureus* compared with *S. chromogenes* IM, *S. chromogenes* TA, and *S. fleurettii* was less pronounced between *S. aureus* and *S. chromogenes* TA (i.e., $0.9 \pm 0.24 \log_{10}$ IL-1β pg/mL; n = 30; $P < 0.05$) than between *S. aureus* and the 2 other CNS-strains (i.e., $1.07 \pm 0.16 \log_{10}$ IL-1β pg/mL for *S. chromogenes* IM, $P < 0.001$; $1.34 \pm 0.15 \log_{10}$ IL-1β pg/mL for *S. fleurettii*, $P < 0.001$; Figure 6B). No significant difference was observed in local IL-1β concentrations between the 2 time points p.i. Notably, a small difference was seen between the CNS- and sham-inoculated glands in their IL-1β concentration (i.e., $0.92 \pm 0.26 \log_{10}$ IL-1β pg/mL; n = 30; $P < 0.05$) for both *S. chromogenes* strains but not for *S. fleurettii* and sham-inoculated glands. The TNF-α levels remained below the quantification limit for CNS-, *S. aureus*-, and sham-inoculated glands. Two out of 6 mice inoculated with *S. chromogenes* TA had a severe local irritation (1 mouse at 28 h p.i. and 1 at 48 h p.i.). In these glands (n = 4), a more pronounced bacterial growth as well as higher local IL-6 and IL-1β concentrations were observed compared with other glands inoculated with the same strain, explaining the high variation for *S. chromogenes* TA in Figure 6A and B. Such distinct differences in response among mammary glands inoculated with the same strain were not observed for *S. chromogenes* IM or *S. fleurettii*. Upon infection with each of the 3 CNS strains, the body

temperature remained normothermic ($36.6 \pm 0.1°C$; n = 21). This was in marked contrast to *S. aureus*-inoculated mice, which initially also remained normothermic until at least 24 h p.i., but became severely hypothermic (drop in body temperature of $7 \pm 1.6°C$; $P < 0.001$; n = 3) within 48 h p.i.

For both experiments the residential microbiota in mammary glands of mice was verified in lactating and sham-inoculated glands. Twenty-three percent of the unchallenged mammary glands were sterile, whereas a limited number of bacterial species were extracted and genotypically identified from the remaining glands: *Staphylococcus lentus* (23%), *Kocuria* spp. (7%), *Bacillus* spp. (7%), *Alcaligenaceae* spp. (15%), *Streptococcus* spp. (15%), and *Microbacterium* spp. (15%; Table 2).

This pilot study explored the usefulness of a previously validated mouse mastitis model for bovine CNS research, which has been mentioned as relevant for human CNS research (Thomsen et al., 1985). A valid mouse model for CNS mastitis would circumvent the
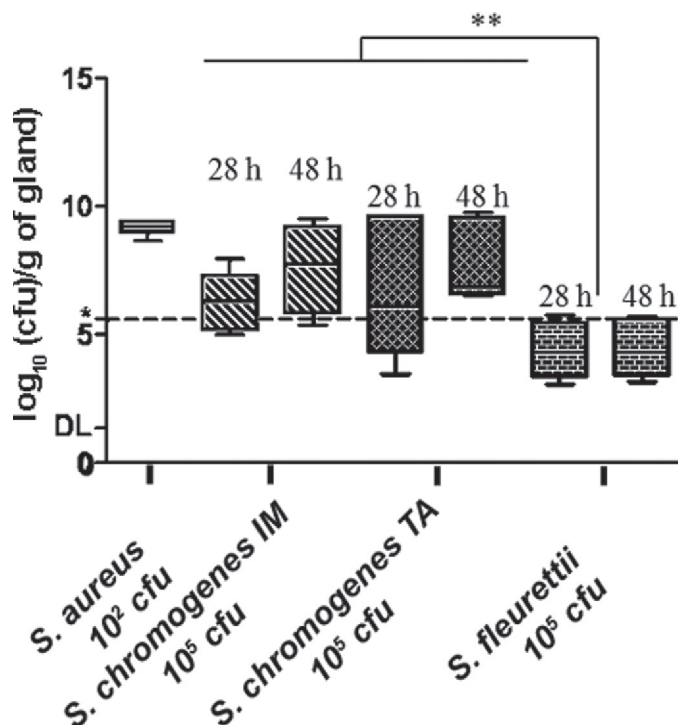

Figure 4. Bacterial growth (expressed as $\log_{10}$ cfu/g of gland) in mammary glands isolated from mice at 48 h postinoculation in experiment 2 and inoculated with *Staphylococcus aureus*, *Staphylococcus chromogenes* intramammary (IM), *Staphylococcus chromogenes* teat apex (TA), and *Staphylococcus fleurettii* after 28 (left box) or 48 h (right box). The line in each box represents the median of these individual data sets. (**$P < 0.01$). The dotted line (*) represents the $\log_{10}$ colony-forming units of CNS per gram of gland injected in the mammary gland of CNS-inoculated mice. The color and pattern code of each group correspond to the one clarified in Figure 1. Error bars represent the standard deviation. DL = detection limit.
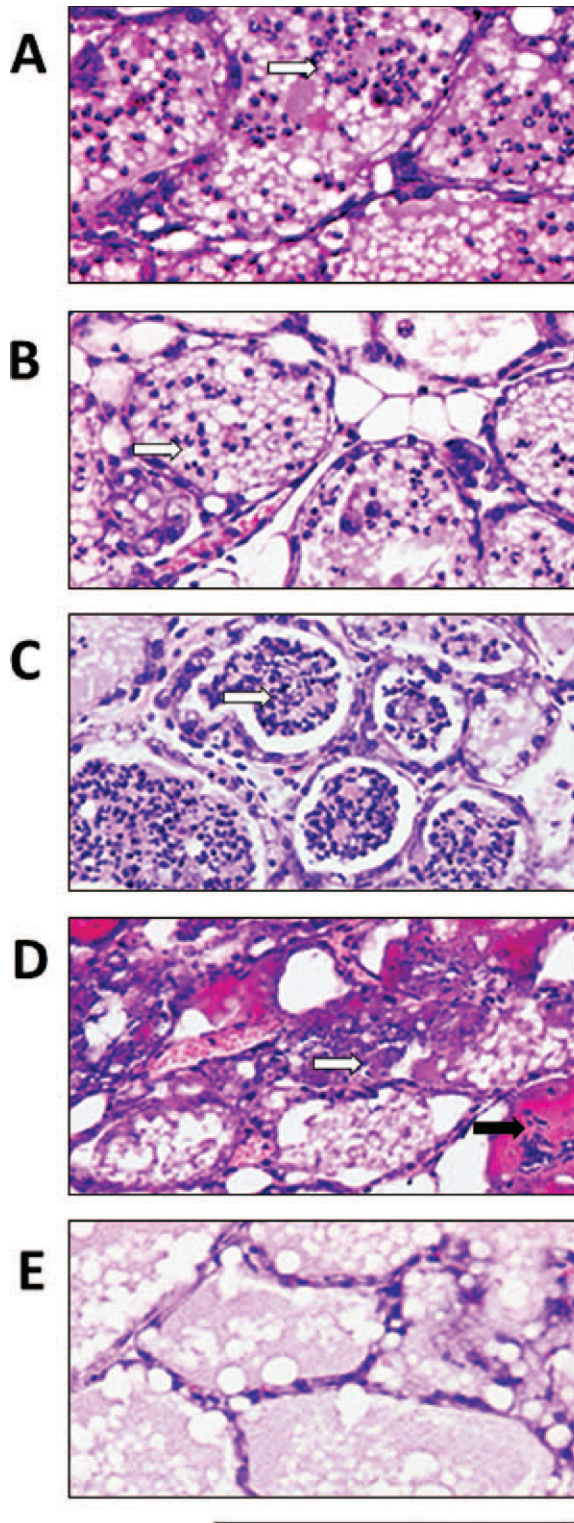
Figure 5. Representative hematoxylin and eosin-stained sections of (A) *Staphylococcus chromogenes* intramammary (IM), (B) *Staphylococcus chromogenes* teat apex (TA), (C) *Staphylococcus fleurettii*, (D) *Staphylococcus aureus*, and (E) PBS/glycerol (sham). The white arrows mark the immune cell content in the alveoli, whereas the black arrows point to the red blood cells in the interstitium. The scale bar represents a length of 200 μm. Color version available online.

major drawbacks of cow challenge experiments. In the first experiment, our data unambiguously displayed limited growth in the murine mammary gland of the bovine CNS isolate that was administered. At 24 h p.i., the glands inoculated with the highest inoculum dose, 10^5 cfu of CNS, were colored red and had a relatively mild alveolar influx of immune cells compared with the mammary glands that were inoculated with *S. aureus.* The latter results corroborate the observations in cows where high inocula of CNS are necessary to obtain colonization in the udder as described for *S. chromogenes*, *S. simulans*, and *S. epidermis* (Simojoki et al., 2009, 2011). Moreover, in contrast to mastitis caused by *S. aureus*, mastitis induced by CNS is less severe and accompanied by mild local signs, such as slight swelling of the udder and color changes in the milk (Taponen and Pyörälä, 2009). Remarkably, sham-inoculated glands were only occasionally sterile. Molecular identification of the bacteria isolated from these glands suggests they originate from the pups, as they are closely related to the commensal microbiota (Martin et al., 2004) from the murine gut and respiratory system (Rodrigue and Lavoie, 1996; Shim et al., 2009; Dimitriu et al., 2013). These bacteria were retrieved in limited number compared with the inoculum and are therefore not considered to have influenced our results to a large extent. Actually, the same species are also isolated from dairy products (i.e., raw milk or cheese; Quigley et al., 2011). To our knowledge, this is the first time that resident bacteria from murine mammary glands were considered in respect to experimentally induced mastitis in this laboratory animal.

In the second experiment, the mouse mastitis model allowed the comparison between 3 CNS isolates differentiated by their anticipated main reservoirs. Again, limited growth of CNS p.i. was observed compared with an *S. aureus* challenge, although each of the CNS strains as well as *S. aureus* caused a neutrophil influx following challenge. Additional cytokine data implied, in general, only a moderate proinflammatory immune response to a challenge with a CNS strain in comparison with *S. aureus*. This corroborated bovine studies, as IL-6 is retrieved following *S. aureus* IMI in contrast to CNS IMI (Hagiwara et al., 2001). Furthermore, the typical gram-negative pathogen-associated induction of TNF-α reported in cows (Bannerman, 2009) and in mice (Notebaert et al., 2008) was also lacking following gram-positive infections in mice. The higher virulence of *S. aureus* versus each of the CNS strains suggested through histology (experiment 1 and 2) and limited cytokine profiling (experiment 2) could be further endorsed with the overall clinical findings in the mice. It should be emphasized that the host innate immune response generated by *S. aureus* challenge was previ-
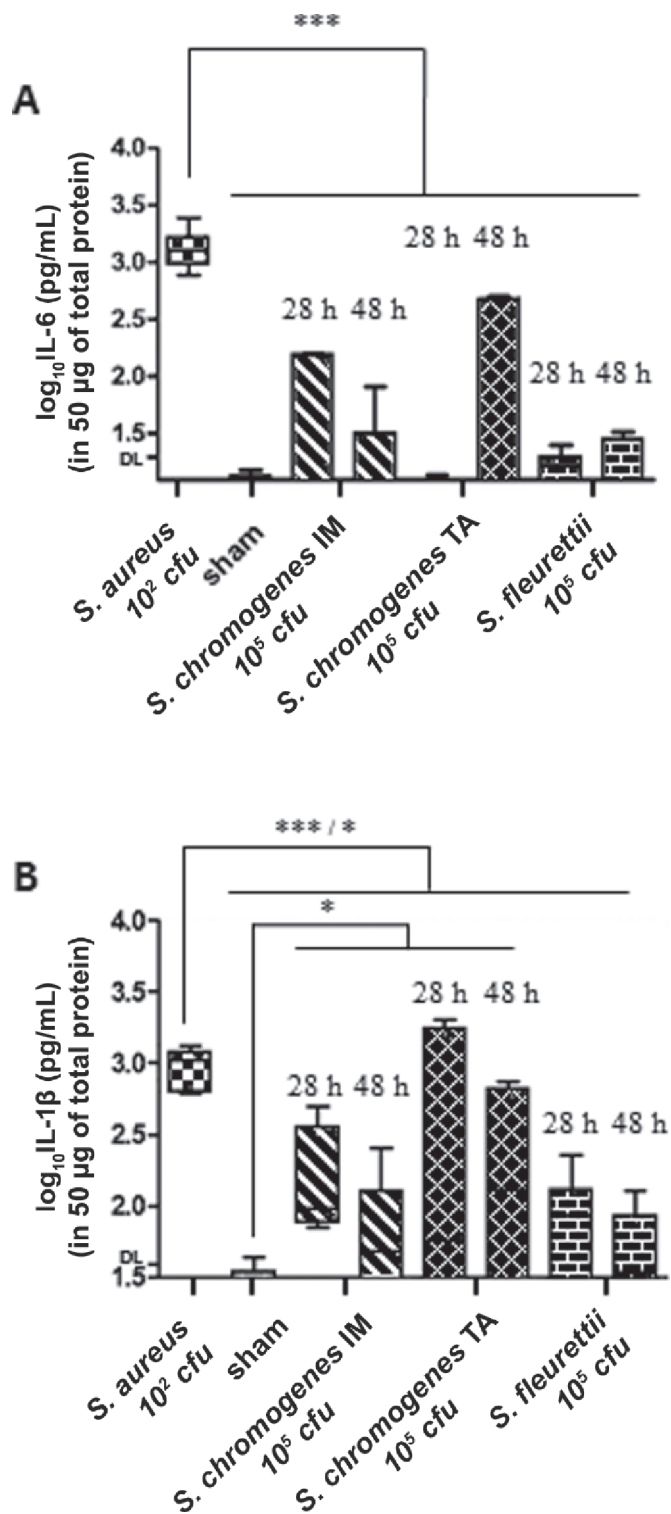
Figure 6. Interleukin-6 (A) and IL-1β (B) cytokine levels in mammary glands isolated from mice of experiment 2 after 28 (left box) or 48 h (right box) inoculated with *Staphylococcus aureus*, PBS/glycerol (sham), and CNS. The line in each box represents the median of these values (***$P < 0.001$; *$P < 0.05$). Error bars represent the standard deviation. The color and pattern code of each group corresponds to the one clarified in Figure 1. IM = intramammary; TA = teat apex; DL = detection limit.

ously reported by several groups (Bramley et al., 1989; Bhakdi and Tranum-Jensen, 1991; Dinges et al., 2000; Brouillette et al., 2004; Chinchali and Kaliwal, 2014) and therefore not unexpected. Our results substantiate the high sensitivity of mice toward the toxins produced by *S. aureus* in this in vivo mastitis model (Jonsson et al., 1985). The absence of coagulase production in transformed *S. aureus* remarkably diminished its intramammary virulence in mice (Jonsson et al., 1985); this most likely also relates to the minimal virulence of CNS in mice at first, as observed in the current study. The lower pathogenicity of the CNS strains compared with *S. aureus* explains the high prevalence of the former pathogens in the milk of clinically healthy cows and their less detrimental effects during bovine mastitis (Pate et al., 2012; Braem et al., 2013; Oikonomou et al., 2014).

Importantly, our data also reflect, at least to some extent, the difference observed in cows between the so-called environmental *S. fleurettii* and the so-called host-adapted *S. chromogenes* IM and *S. chromogenes* TA. Corroborating our bacterial growth, cytokine level data, the discriminating grouping of presumed neutrophils in the murine mammary gland sections, and the low prevalence in milk samples of cows allowed to speculate that *S. fleurettii* is able to colonize the milk habitat but is less likely to multiply locally compared with both *S. chromogenes* strains (Piessens et al., 2011; De Visscher et al., 2014). Although the number of animals should be increased to allow for a precise interpretation of the statistical relevance of our results, these suggest an underlying difference in the hosts' innate immune response between *S. chromogenes* IM and *S. chromogenes* TA. More specifically, the *S. chromogenes* IM strain might be more adapted to the intramammary niche (milk environment) and as such induce less pathophysiological effects compared with the likely more teat-adapted *S. chromogenes* TA.

In conclusion, our study suggests that, as previously demonstrated for *S. aureus*, the mouse model can be an attractive complementary in vivo tool for bovine CNS mastitis research while minding the limitations, such as the presence of a commensal murine mammary gland microbiota. Nevertheless, overall it mimics the mild inflammation in cows induced by an IMI with CNS through a neutrophil influx accompanied either with or without a low induction of certain proinflammatory cytokines. Moreover, the limited data of our pilot study tentatively suggest that the CNS mouse model can distinguish the differences in host response inflicted by different strains of the CNS group. Future research is warranted and should include infection studies with longer duration besides the comparison of even more bovine CNS isolates. This would allow drawing reliable conclu-

Table 2. Residential microbiota in the mammary gland of mice

Treatment[1] and gland[2]	Replicate	Dilution	No. of colonies[3]	Result
Lactating				
1	A	1	—	—
1	B	1	1	*Staphylococcus lentus*
2	A	1	1	*Staphylococcus lentus*
2	B	1	1	*Staphylococcus* spp.
3	A	1	1	*Kocuria* spp.
3	B	1	—	—
4	A	1	—	—
4	B	1	—	—
5	A	1	2	*Microbacterium* spp.
5	B	1	1	*Microbacterium* spp.
6	A	1	1	*Bacillaceae*
6	A	1	1	*Staphylococcus lentus*
6	B	1	1	*Staphylococcus lentus*
7	A	1	—	—
7	B	1	—	—
8	A	1	—	—
8	B	1	—	—
9	A	1	—	—
9	B	1	2	*Microbacterium* spp.
Sham				
1	A	1	—	—
1	B	1	1	*Streptococcus* spp.
1	A	2	55	*Streptococcus* spp.
1	B	2	73	*Streptococcus acidominimus*
2	A	1	6	*Streptococcus acidominimus*
2	B	1	5	*Streptococcus* spp.
2	A	2	31	*Streptococcus acidominimus*
2	B	2	49	*Streptococcus acidominimus*
3	A	1	31	*Alcaligenaceae*
3	B	1	38	*Alcaligenaceae*
3	A	2	5	*Alcaligenaceae*
3	B	2	6	*Alcaligenaceae*
3	A	1	22	*Alcaligenaceae*
4	B	1	20	*Alcaligenaceae*
4	A	2	2	*Alcaligenaceae*
4	B	2	2	*Alcaligenaceae*

[1]Lactating mammary glands were not injected and not weaned from the litter; sham-inoculated glands were weaned from the litter for at least 24 h before inoculating with PBS.

[2]Different numbers represents different glands.

[3]The number of colonies with a distinct phenotype compared with other colonies.

sions on the specific differences between CNS species and strains, as observed in cows. Moreover, analyzing the influence of the mouse strain would be valuable for the in-depth validation of our mouse mastitis CNS model. Finally, this mouse model is potentially also an elegant tool to explore the inflammatory factors including cytokines that are enhanced by CNS when inducing a local neutrophil influx.

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