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A comparison between the immune response in macrophages when exposed to two different strains of *Streptococcus agalactiae*

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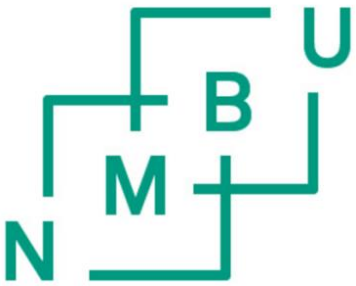

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List of publications

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Abbreviations and glossary

BSA	Bovine serum albumin
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
DAMP	Damage-associated molecular pattern
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phospate dehydrogenase
GPCRs	G-protein-coupled receptors
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-1β	Interleukin-1 beta
IFN-γ	Interferon gamma
LPS	Lipopolysaccharides
LB-broth	Luria broth
M1	Classically activated macrophages
M2	Alternatively activated macrophages
mRNA	Messenger RNA
miRNA	Micro RNA
MOI	Multiplicity of infection
NMBU	Norwegian University of Life Sciences
NR	Norwegian Red Cattle
OD	Optical Density
PBMC	Peripheral blood mononuclear cells
PAMP	Pathogen-associated molecular pattern
RBC	Red Blood cell
RIN	RNA integrity numbers
RPMI	"Rosewell Memorial Park Institute" medium
RT-qPCR	Reverse transcription-quantitative Polymerase Chain Reaction
SCC	Somatic cell count
TGFβ1	Transforming growth factor beta 1
TH broth	Todd Hewitt Broth
TLR	Toll like receptor
TLR2	Toll like receptor 2
TNF-α	Tumor necrosis factor alpha

Abstract of thesis

Mastitis is the most common disease in the dairy industry today. In the mammary gland, macrophages play a leading role in the immune response as first line defence against invading udder pathogens. Different candidate genes associated with the response against udder pathogens are detected, where some of these candidate genes represent pro-inflammatory cytokines produced by macrophages. However, recent studies from our group indicate that macrophages infected with bacteria such as *Staphylococcus aureus* also produce anti-inflammatory cytokines in an alternative response. The alternatively activated macrophages counteract many pro-inflammatory mechanisms, and this may be a strategy for udder pathogens to evade the host immune response. A key question is whether macrophages exposed to *Streptococcus agalactiae* will have a greater inclination towards alternative than classical activation, and if this diversify between the different sequence types (ST) of the bacteria. We investigated the early phase response of bovine monocyte-derived macrophages infected in vitro with two different sequence types of live *Streptococcus agalactiae* (ST103 and ST12) *in vitro*, by examining the transcription level of six macrophage-associated cytokines. First, we isolated monocyte-derived macrophages from six healthy Norwegian Red (NR) cows aged 2,5-7 years, and for each individual animal the immature macrophages were divided into four classes. Two classes were infected *in vitro* with either of the two *S. agalactiae* strains with a multiplicity of infection (MOI) of 1, then incubated for 1 hour before penicillin/streptomycin was added in each well, and further incubated for 5 hours (a total of 6 hours). The third cell class was exposed to Lipopolysaccharides (LPS) (positive control) and the last class was left uninfected (negative control), and both positive and negative control were treated equally as the infected cells with penicillin/streptomycin and incubated for a total of 6 hours. Originally, we planned to compare the early phase response also with monocyte-derived macrophages infected with *Staphylococcus aureus in vitro*, but we were not able to continue this work due to a non-reproducible method when infecting the cells with *S. aureus*. Consequently, this part of the study was abandoned.

Further we isolated total RNA from the cells infected with *S. agalactiae* ST12 and ST103, and measured the transcript levels of *Tumor Necrosis Factor α (TNFα), Interleukin 1β (IL-1β), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 10 (IL-10) and Transforming growth factor β1 (TGFβ1)* by Reverse transcription-quantitative Polymerase Chain Reaction (RT-qPCR).

TNF-α, *IL-1β*, *IL-6*, *IL-8* and *IL-10* were significantly up-regulated by ST12, ST103 and LPS compared to the negative control. *IL-6* and *IL-10* displayed different responses both between ST103 and LPS, and between ST12 and LPS, with high levels of IL-10 (anti-inflammatory cytokine) and low levels of IL-6 (pro-inflammatory cytokine) in the cells infected with *S. agalactiae* compared to cells activated by LPS. *TGFβ1* were significantly down-regulated only in the macrophages infected with ST12. When comparing the transcription levels of the cytokines between the macrophages infected with the two strains of *S. agalactiae*, we did not observe significantly different expression of any of the six cytokines. Thus, we know there is activation of both pro-inflammatory and anti-inflammatory cytokines when infected with *S. agalactiae,* and that there is a decrease in the anti-inflammatory signal of *TGFβ1* only in macrophages infected with ST12. We also propose that the macrophages infected with bacteria might be activated in the alternative pathway compared to macrophages activated by LPS, but this field of study needs further investigation.

Background

Mastitis is a common and costly disease among dairy cows in Norway (Halasa, Huijps, Osteras, & Hogeveen, 2007). It is one of the main reasons for the use of antibiotics in dairy farms, impairs the welfare of the cow and affects the milk production. Mastitis is a multifactorial disease, where the load and pathogenicity of the infecting microbe is one of the influencing factors, as well as milking hygiene, the immune status and the genetics of the animal.

Staphylococcus aureus is still the major pathogen causing mastitis (Persson Waller, Bengtsson, Lindberg, Nyman, & Ericsson Unnerstad, 2009), but *Streptococcus agalactiae* has re-emerged as a disease causing agent, especially in dairy farms with automatic milking systems and large herds (Katholm, 2010). The recent modernization of the dairy industry in Norway might be influencing this, where the herd sizes and milk production have increased, and there have been changes in housing and milking systems. In a national report from 2012 it was presented that the prevention of infection with *Str. agalactiae* is an important research area for the Norwegian dairy industry (NVH, 2012).

It has been indicated in a collaboration project with EU NoE EADGENE that there is a connection between the animals genetics and the immune response against mastitis (Genini et al., 2011) (Lewandowska-Sabat, Gunther, Seyfert, & Olsaker, 2012). Different genome regions, candidate genes and pathways have been associated with the response against the udder pathogens. Some of these candidate genes represent pro-inflammatory cytokines produced by macrophages. However, recent studies from our group indicate that macrophages infected with bacteria such as *S. aureus* also produce anti-inflammatory cytokines in an alternative response. (Lewandowska-Sabat et al., 2013).

1.1 The role of macrophages at inflammation

A major way that the immune system handles infections is to stimulate acute inflammation by accumulating and activating leukocytes and plasma proteins at sites of infection. Macrophages are phagocytes and a part of the innate immune system, and are critical effectors and regulators of inflammation and serve as a first line defense against invading pathogens. They are found in

all tissues in adult mammals, and display great diversity in their functions (A.K. Abbas, A.H. Lichtman, & S. Pillai, 2015).

When a pathogen invades the udder, tissue macrophages among other cells will respond by recognizing pathogen-associated molecular patterns (PAMPs). PAMPs are structures produced by microbial pathogens and not by mammalian cells, and macrophages are equipped with a broad range of pathogen-recognition receptors that will recognize PAMPs (Geissmann et al., 2010) . The response to PAMPs will stimulate production of pro-inflammatory mediators, that further recruit neutrophils and blood monocytes to the site of infection. (Arango Duque & Descoteaux, 2014; Bannerman et al., 2004; Boulanger, Bureau, Melotte, Mainil, & Lekeux, 2003). The newly arrived blood monocytes mature into tissue macrophages, and ingest microbes into vesicles by phagocytosis and destroy them, and will simultaneously produce and secrete more cytokines. (Mullan, Carter, & Nguyen, 1985) The macrophages also participate in remodeling of the infected or injured tissue, by converting to produce anti-inflammatory mediators and growth factors for endothelial cells and fibroblasts. (Rainard & Riollet, 2006; Wynn, Chawla, & Pollard, 2013).

1.2 Cytokines

Cytokines are a large heterogeneous group of secreted proteins that regulate and coordinates the activity of cells in all aspects of the immune system. All immune cells secrete some cytokines and express receptors for several cytokines. The cytokines play a role in differentiation and growth of immune cells, activation of effector functions of phagocytes and lymphocytes at inflammation, infection or tissue damage, and they direct movement of leukocytes both from blood into tissues and within tissues. The cytokines originating from the innate immune system are mainly produced by macrophages and dendritic cells, and most of them have paracrine action on cells close to their origin. Macrophages produce different types of cytokines like pro-inflammatory and anti-inflammatory cytokines based on the type of stimuli before activation. The pro-inflammatory cytokines produced by activated macrophages (e.g. *TNF-α, IL-1β*, *IL-6*, and *IL-8)* are involved in the acute inflammatory response, whereas anti-inflammatory cytokines like *IL-10* are involved in limiting the inflammatory response. Some cytokines like *TGFβ1* have actions that are both regulatory, inflammatory and anti-inflammatory depending on the context,

and are also involved in tissue repair (Chockalingam, Paape, & Bannerman, 2005; Schukken et al., 2011) .

1.2.1 *TNF-α* – Tumor necrosis factor alpha

TNF- α is a mediator of the acute inflammatory response to bacteria and other microbes. It was first discovered in 1975 as a molecule causing necrosis in tumors. (Carswell et al., 1975). This is now known to be the result of inflammation and thrombosis of the blood vessels in the tumor, but the name from the discovery persists. *TNF- α* is an important protein that are involved in regulating proliferation and cell differentiation, apoptosis and inflammatory gene expression. It is produced by macrophages and dendritic cells among others, and the production in macrophages is stimulated by many different microbes by PAMPs and DAMPs. LPS from the cell wall of gram-negative bacteria is one example of a ligand that binds Toll-like receptors (TLRs) and stimulates production of *TNF- α.* (Arango Duque & Descoteaux, 2014).

1.2.2 *IL-1β* – Interleukin 1 beta

IL-1β is also one of the most important pro-inflammatory cytokines and a mediator of the acute inflammatory response, and shares several actions with *TNF- α* like regulating cell proliferation, cell differentiation and apoptosis. It is one of the most potent endogenous inducers of fever. (Dinarello, 1998). *IL-1β* is produced and secreted as mature proteins mainly in monocytes and macrophages, and circulates systemically (Dinarello, 1996). *IL-1β* mediates its effect by a membrane receptor expressed on many cells like leukocytes, epithelial and endothelial cells. Production of *IL-1β* is stimulated by a two-step activation; first an activation of a new gene transcription and production of the polypeptide, followed by another signal that activates a proteolytic inflammasome to cleave the precursor into the mature protein. (Arango Duque & Descoteaux, 2014).

1.2.3 *IL-6* – Interleukin 6

Interleukin 6 is a pleiotropic cytokine with both pro- and anti-inflammatory properties, and is important in acute inflammatory responses and have both local and systemic effects. It is secreted by macrophages and T-cells among others, and the expression is induced by bacteria, viruses and other cytokines (*TNF-* α, IL-1 β) (Biffl, Moore, Moore, & Peterson, 1996). The

cytokine has many functions, including stimulating the production of multiple chemokines, increasing the production of antibodies in activated B-cells and it is involved in production of acute phase proteins (APPs) and other inflammatory mediators in the liver. (DeVito & D'Áquino, 2013; Fattori et al., 1994). It also promotes the differentiation of *IL-17* producing T_H17-cells (Acosta-Rodriguez, Napolitani, Lanzavecchia, & Sallusto, 2007).

1.2.4 *IL-8* – Interleukin 8

Interleukin 8 was identified in 1987 as a novel type of cytokine that activated neutrophils. (Baggiolini, Walz, & Kunkel, 1989). It is now known as the major chemokine supporting neutrophil chemotaxis, and is also named CXCL8. The cytokine is produced by tissue macrophages among other cells, in response to bacteria, viruses, fungi, parasites as well as other cytokines like *TNF-α* and *IL-1β* (Matsushima et al., 1988), and is responsible for causing neutrophils to migrate into the affected tissues, activating the cells and stimulating phagocytosis. (Baggiolini & Clark-Lewis, 1992; Nishimura, 2003).

1.2.5 *IL-10* – Interleukin 10

Interleukin 10 are involved in control of the innate immune reactions and cell mediated immunity by inhibiting activated macrophages and dendritic cells. It is produced in many immune cells, including monocytes (de Waal Malefyt, Abrams, Bennett, Figdor, & de Vries, 1991), activated macrophages (Fiorentino, Zlotnik, Mosmann, Howard, & O'Garra, 1991), T_H1 cells, T_H2 cells (Del Prete et al., 1993) and dendritic cells, and regulates its production by negative feedback. *IL-10* inhibits the production of *IL-12*, and thereby controls *IFN-γ* secretion, and inhibits T-cell activation and terminates cell-mediated immune reactions. (D'Andrea et al., 1993; Mittal & Roche, 2015; Taga, Mostowski, & Tosato, 1993; Taga & Tosato, 1992)

1.2.6 *TGFβ1*- Transforming growth factor beta

Transforming growth factor beta 1 was first discovered as a peptide with an ability to induce phenotypic transformation of non-neoplastic cells *in vitro*. (Roberts, Frolik, Anzano, & Sporn, 1983). *TGFβ1* is now known to have many functions; for instance, inhibiting the activation of macrophages and the proliferation and effector functions of T-cells, promoting tissue repair by affecting collagen synthesis, angiogenesis and fibrosis, and stimulating production of IgA

antibodies from B cells. It is also suspected that the cytokine work as a direct chemotactic signal to monocytes at low concentrations.(Wahl et al., 1987). During pregnancy *TGFβ1* is required in the mammary gland for alveolar development and functional differentiation, and inhibiting secretion of milk proteins until parturition. (Daniel, Robinson, & Silberstein, 2001). *TGFβ1*is produced by activated macrophages, regulatory T-cells among other cell types. It is synthesized as an inactive precursor, and further cleaved in the Golgi plexus from proteolytic activity before the mature *TGFβ1* are secreted together with other polypeptides that need to be removed extracellularly before the cytokine can bind to receptors (Gleizes et al., 1997; Travis & Sheppard, 2014).

1.3 Activation of macrophages

The presence of microbes together with the host microenvironment determinates the activation of macrophages (Sica, Erreni, Allavena, & Porta, 2015). When the cells at the site of infection respond to the microbe; activated T_H1-cells and Natural killer cells (NK-cells) secrete *IFN-γ*, that is the major cytokine responsible for activating macrophages (Dalton et al., 1993). The secretion of *IFN-γ* by NK-cells is a part of the innate immune system, and together with Toll-like receptor signals from microbes it will activate the macrophages. The T_H1-cells activates macrophages both from secretion of *IFN-γ* and directly by contact-mediated CD40-ligand interactions - as a part of adaptive immunity. The activated macrophages secrete pro-inflammatory cytokines and get increased microbicidal activity by increasing the production of reactive oxygen species, nitric oxide and the lysosomal enzymes. This activation of macrophages from *IFN-γ* is called "classical macrophage activation" (M1). (A.K Abbas, A.H Lichtman, & S Pillai, 2015). .

However, the macrophages can also be activated in an alternative way (M2); by *IL-4* and *IL-13* produced generally in T_H2-type responses. The alternative activation are less well defined, but M2-cells express enzymes that promote collagen synthesis and fibrosis [e.g. *TGFβ1*] that induce scarring and fibroblast proliferation, and they produce anti-inflammatory cytokines [e.g. *IL-10*] that inhibits the pro-inflammatory cytokines produced by the classically activated macrophages. (Gordon, 2003). Recently the M2 macrophages have been further divided into three different subsets based on their type of activation; M2a, M2b and M2c. M2a-cells are induced by *IL-4* and *Il-13* and are involved in type II inflammation and T_H2-type responses, M2b-cells are induced by

immune complexes and agonists of TLRs or IL-1R and are and less understood but thought to have immunoregulatory functions, and M2c; stimulated by *IL-10* and glucocorticoids and involved in suppressing immune responses and tissue remodeling (Avdic et al., 2013), (Mantovani et al., 2004).

The balance between pro- and anti-inflammatory signals is crucial for immune regulation of the inflammation and preventing chronic conditions. In the bovine mammary gland, the status of differential macrophage activation may be pivotal for the defense and resolution of mastitis (Duvel, Frank, Schnapper, Schuberth, & Sipka, 2012).

1.4 Mastitis

Mastitis is an inflammation in the mammary gland, usually a result of microbial infection, where bacteria like *Staphylococcus aureus, Escherichia coli* and *Streptococcus agalactiae* are common pathogens. (Watts, 1988). In 1917, Breed and Brew identified the first *Streptococcus ssp.* from an infected udder as a cause of high bacterial count in the milk (Breed & Brew, 1917).

The udder has different barriers to prevent that microbes invade and infect the tissue. The teat skin works as a physical barrier against the microbes, and small injuries in the skin could make a passage for pathogens into the udder. Another important defense mechanism is the teat canal occluded with keratin; the significance of this keratin lining was discovered by Murphy et al in 1959 who showed *in vivo* that removing some of the soft keratin from the teat canal temporarily destroyed the barrier function against *Streptococcus agalactiae* (Murphy, 1959). In addition to the physical barrier, keratin contain proteins that functions as a chemical barrier and are shown to inhibit growth of two strains of *Staphylococcus aureus* and one strain of *Streptococcus agalactiae. (Hibbitt, Cole, & Reiter, 1969).* If the barrier with the skin and keratin is unsuccessful, the next defense mechanism against invading microbes is the inflammation process. Factors like age, stage of lactation, the animal´s immune status and genetics combined with the pathogen species, strain and virulence mechanism will determine the inflammation response.

As mentioned earlier, macrophages play a role in the first line defense against invading pathogens. The polarization of the tissue macrophages influence the immune response against microbes, but there is dim knowledge of the distribution and importance of the phenotypes of macrophages in the udder. In a study from 2013 our group concluded that macrophages infected with mastitis causing bacteria such as *S. aureus* also produce anti-inflammatory cytokines in an alternative response, and that this alternative activation could be contributing to intracellular persistence of *S. aureus*. (Lewandowska-Sabat et al., 2013)

Clinical mastitis is characterized by clinical symptoms such as perceptible udder inflammation, often with secondary depression, anorexia and fever, and could be either acute or chronic. However, the infection of udder pathogens can also result in chronic subclinical mastitis; with no clinical observable signs but identified by changes in milk composition like increased somatic cell count (SCC).

The Norwegian Cattle Health Recording System records health and production data of each individual cow in Norway, including veterinary treatments and diagnoses. This data is further used for research, breeding guidance and heard health management among other purposes, and gives valuable information about the prevalence of mastitis and udder health in the Norwegian dairy cattle population. SCC is often used as an indicator of udder health, as the SCC increases during the inflammation process in the udder (Harmon, 1994). A healthy udder usually have a SCC below 50,000 cells/mL (Barbano, Ma, & Santos, 2006), but the normal healthy mammary gland is often defined with a SCC below 100,000 cells/mL. (Schwarz et al., 2011). The somatic cell composition is known to be lymphocytes, macrophages and mostly polymorphonuclear leukocytes (PMN). (Piccinini, Bronzo, Moroni, Luzzago, & Zecconi, 1999; Schwarz et al., 2011). High SCC is defined as more than 200,000 cells/mL, this threshold value is often used to characterize subclinical mastitis. (Dohoo & Leslie, 1991; Harmon, 1994; Pantoja, Hulland, & Ruegg, 2009; Schepers, Lam, Schukken, Wilmink, & Hanekamp, 1997). The Norwegian Cattle Health Recording System have recorded the SCC in bulk milk since 1978, and the farmer get merit bonuses if the geometrical average on the previous six milk samples have a cell count below 230,000 cells/mL, known as Premium milk (Osteras et al., 2007; TINE, 2018).

In Norway, a selection program has been active for more than 20 years, wherein two subpopulations of Norwegian Red cows strongly selected for high protein yield or low mastitis incidence have been bred. Proven sires from the breeding program of Norwegian Red cattle are used, and are mated to cows in the same subpopulation every 3-4 year. It was observed an unfavorable genetic correlation between mastitis and milk production, which suggests that the selection for increased milk production result in a decrease in the resistance against mastitis, and also against other diseases like ketosis and retained placenta. (Heringstad, Klemetsdal, & Steine, 2007).

1.5 Staphylococcus aureus

S. aureus is a Gram-positive coccus, that is both a commensal and pathogen of several animal species, including cattle and humans. It causes both clinical and subclinical mastitis, and possesses about 200-300 virulence factors that make the bacteria able to adhere and invade host cells, and escape the immune response. (Fraunholz & Sinha, 2012). The virulence factors can be activated or supressed depending on the situation, and by that result in different phenotypes within the same strain of bacteria. In bovine mastitis, the most prevalent virulence factors include genes encoding 4 haemolysins (α, β, λ, and δ), leucocidin components, clumping factors, fibrinogen binding protein and fibronectin-binding protein A. (Artursson, Söderlund, Liu, Monecke, & Schelin, 2016, p.156-161; Dinges, Orwin, & Schlievert, 2000). These factors are involved in escaping from phagosomes, adhesion, lysing cells, chemotaxis and other functions that aims to target the immune cells and survive intracellularly (Vandenesch, Lina, & Henry, 2012). The bacteria causing mastitis are mainly located in the udder and is transmitted between animals directly from contact, and indirectly usually related to the milking process. (Dodd, 1983). When entering the teat canal the bacteria colonises and adhere to the epithelial cells, and it seems that the closer to the teat sinus the colonisation occur, the higher are the risk of developing mastitis. (Prasad & Newbould, 1968).

1.6 Streptococcus agalactiae

S. agalactiae is a Gram-positive coccus, also known as group B streptococcus because of its Lancefield group B antigen. It is a major cause of neonatal sepsis and meningitis in humans, and are asymptomatically carried by a large proportion of adults particularly in the urinary tract and

gastrointestinal tract (Lyhs et al., 2016). In cattle *S. agalactiae* colonizes the milk ducts and causes mastitis. The intramammary infections are often chronic and subclinical, with intermittent incidents of clinical mastitis. (Quinn et al., 2011). Moderately elevated SCC and reduced milk production are often the only symptoms of mastitis caused by *S. agalactiae* (Eberhart, Hutchinson, & Spencer, 1982). Consequently unidentified infected animals often function as a reservoir of the pathogen. (Keefe, 1997). *S. agalactiae* have different virulence mechanisms to manage a successful invasion of the host animal. It resists phagocytosis by an antiphagocytic capsule (Orefici, Recchia, & Galante, 1988; Rubens, Wessels, Heggen, & Kasper, 1987), are able to survive intracellularly in macrophages, (Cornacchione et al., 1998; Valentin-Weigand, Benkel, Rohde, & Chhatwal, 1996), and have the ability to kill macrophages by inducing apoptosis. (Fettucciari et al., 2000)

S. agalactiae seems to be difficult to eliminate when first established in the herds. Recently "The National Veterinary Institute" in Norway collected data from infected herds and identified reasons for the failing control of the bacteria. Different sequence types of *Str. agalactiae* were detected, and they seem to act differently in the environment and differ in grade of pathogenicity (Jorgensen et al., 2016). ST103 was detected with considerable environmental occurrence, while ST12, one of the strains usually associated with colonization of pregnant women, was found in cattle herds with no positive environmental samples. ST103 is also reported among bovine isolates from Denmark and Eastern China (Yang et al., 2013; Zadoks, Middleton, McDougall, Katholm, & Schukken, 2011).

Aims

The primary objective of this study is to increase the understanding of the immune response related to different udder pathogens of current interest causing intramammary infections, on the basis of the previous research performed by our group (Lewandowska-Sabat et al., 2013), The Norwegian Veterinary Institute (Jorgensen et al., 2016) and Heringstad *et al (Heringstad et al., 2007).* The main focus will be on the macrophage response when the cells are exposed to two different sequence types of *S. agalactiae* (ST103 and ST12) that seem to differ in environmental distribution and pathogenicity and are related to subclinical mastitis, and compare this immune response to the response against *S. aureus* that is already investigated (Lewandowska-Sabat et al., 2013).

This research program is associated to "Multimast"; a collaboration project that proposes a more overall picture of bovine mastitis, where the bacteria, immune response and host genetic background are considered simultaneously. This might give a deeper understanding of the pathogenesis in mastitis and the immune response when the udder is infected with *S. agalactiae.* Recent studies by our group indicate that macrophages also produce anti-inflammatory cytokines in an alternative response to infection with bacteria such as *S. aureus*. (Lewandowska-Sabat et al., 2013). Since the alternatively activated macrophages acts to counteract many inflammatory mechanisms this may be a strategy for the bacteria to evade the host immune response and survive intracellularly. *S. agalactiae* is related to chronic subclinical mastitis infections and are known to be able to survive intracellularly in macrophages, and a key question is whether macrophages exposed to *S. agalactiae* will have a greater inclination towards alternative than classical activation.

We hypothesised that there is a difference in the expression of candidate genes (cytokines) in bovine monocyte-derived macrophages infected with ST103, ST12 and *S. aureus in vitro,* and that the immune response in the macrophages exposed to *S. agalactiae* would have a shift towards the alternatively activating pathway. These measurements are considered in relation to an additional study where we investigated the expression of microRNA in the macrophages infected with *S. agalactiae* (ST103 and ST12), that are hypothesized to be key regulators of the cytokine responses.

Abstract of appended publication

MicroRNA expression profiles of bovine monocyte-derived macrophages infected *in vitro* with two strains of *Streptococcus agalactiae* (Lewandowska-Sabat et al., 2018)

Background: MicroRNAs (miRNAs) are short, non-coding RNAs that regulate gene expression at the post-transcriptional level and play a key role in the control of innate and adaptive immune responses. For a subclinical infection, such as bovine streptococcal mastitis, early detection is a great challenge, and miRNA profiling could potentially assist in the diagnosis and contribute to the understanding of the pathogenicity and defense mechanisms. We have examined the miRNA repertoire and the transcript level of six key immune genes [tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and transforming growth factor beta 1 (TGFβ1)] during the early phase response of bovine immature macrophages to in vitro infection with live Streptococcus agalactiae. Next generation sequencing of small RNA libraries from 20 cultures of blood monocyte derived macrophages exposed to either one of two sequence types of S. agalactiae (ST103 or ST12) for 6 h in vitro and unchallenged controls was performed.

Results: Analyzes of over 356 million high quality sequence reads, revealed differential expression of 17 and 44 miRNAs ($P < 0.05$) in macrophages infected with ST103 and ST12, respectively, versus unchallenged control cultures. We also identified the expression of 31 potentially novel bovine miRNAs. Pathway analysis of the differentially regulated miRNAs and their predicted target genes in the macrophages infected with ST12 revealed significant enrichment for inflammatory response and apoptosis, while significant enrichment for integrin and GABA signaling were found in ST103 infected macrophages. Furthermore, both bacterial strains regulated miRNAs involved in the alternative activation of macrophages. The transcript levels of TNF-α, IL-1β, IL-6, IL-8 and IL-10 were significantly up-regulated by both bacterial strains, however the expression of TGFβ1 was significantly down-regulated only by ST12.

Conclusions: Our study identified pathogen-induced differential regulation of miRNAs controlling inflammation and polarization in bovine macrophages. This implies that miRNAs have potential to serve as biomarkers for early detection of bacterial infection.

My contribution to the submitted paper, project and research

During my year in the research program I was involved in both planning and performing of the study featured in the submitted paper, and my contribution to this article is elaborated more detailed this thesis. I participated in the design of the study, was responsible for preparation and batching of the bacterial strains, carried out the isolation of monocyte-derived macrophages, the cell infection experiments and the RNA-isolation in the lab. I also performed the cDNA synthesis and RT-qPCR analysis. I did not participate substantially in aspects related to the miRNA analyses.

During the research program, I also contributed in other lab experiments performed in our research group. In the study published by Hege Lund et al, I was involved in blood sampling and injection of adjuvant in the animals, post-mortem collection of sample material, isolation of peripheral blood mononuclear cells (PBMC), and the flow cytometry analysis. (Lund, Boysen, Akesson, Lewandowska-Sabat, & Storset, 2016).

Materials and methods

Animals and cell isolation

Six healthy NR cows aged 2,5-7 years were used for the experiments. The animals were maintained under uniform housing, environmental and nutritional conditions at the Norwegian University of Life Sciences. The blood was sampled by certified personnel and conducted in agreement with the provisions enforced by the Norwegian Research Animal Authority.

500 ml of blood was collected from the jugular vein of each animal in sterile glass bottles with sodium citrate as anticoagulant. Peripheral blood mononuclear cells (PBMC) were extracted on Lymphoprep™ (Axix-Shield, Norway) by density gradient centrifugation; 2210xg for 30 minutes. The cell layer was transferred into new tubes and washed repeatedly with PBS containing 2mM EDTA. Remaining RBC were lysed by adding cold and sterile dH_2O for 30 seconds, then adding double concentrated PBS with EDTA. The cells were counted and the viability evaluated with Countess® Automated cell counter (Invitrogen) following the manufacturer's instructions.

CD14+-cells were extracted by positive selection of monocytes with antigen CD14, using anti-human CD14 MACS® MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Preparation for extraction was done by adding 80 µl PBS with 2mM EDTA and 0,5 % BSA per 10^7 cells, and 10 µl MACS® MicroBeads per 10^7 cells. The cells were filtered through a 70µm cell strainer (Falcon, Corning® Inc. – Life Sciences, One Becton Circle, Durham, USA) to avoid lumping and maintain a uniform cell suspension. $0,5x10^6$ cells were saved for flow cytometry before the extraction. The cell isolation was done by using two MACS®columns placed in MACS® Separators, where a strong magnetic force within the column retains cells labelled with the beads. The CD14+cells from the columns were washed multiple times with PBS with 2mM EDTA and 0,5% BSA. Further the cells were eluted into a new tube and added 5 ml RPMI medium. The cells were counted again with Countess® Automated cell counter (Invitrogen), and $0,5x10^6$ cells were spared for flow cytometry. The remaining cells were washed with PBS with

0,5% BSA, before separated into 6-well dishes with a density of 1,5 x 10^6 cells pr well, adding 3 ml RPMI medium supplemented with 10% FCS (Invitrogen, Carlsbad, USA) into each well.

Cells were incubated over night at 37C° in an atmosphere with 5% CO2 before infection, to sustain the early-stage macrophages during infection and simulate the early phase response from the animal. The growth media did not contain antibiotics to minimize the stress on the cells by avoiding excessive washing and to evade interaction when infecting the cells. The phenotypic morphology of cell differentiation of monocytes into an early-stage macrophage phenotype was confirmed by phase contrast microscopy.

Flow-cytometry

The purity of selected CD14+ cells was verified by flow cytometry, analysing in a Gallios flow cytometer (Beckman Coulter). The cells saved pre-isolation and the positively selected cells were stained directly with secondary colouring PE conjugated anti-mouse IgG2a (Southern Biotech, Birmingham, Al, USA). The data was analysed in Kaluza software (Beckman Coulter), and the purity was found to be in the range of 95-98%.

Bacterial growth

As preparation for multiplication of the bacteria; LB-broth, TH-broth and 20% Glycerol broth were prepared. LB-broth were made by adding 8 g Tryptone, 4 g yeast extract and 7,2g sodium chloride into 800 ml distilled water. TH-broth powder (Sigma Aldrich, Darmstadt, Germany) consists of 500 g/l beef-heart infusion, 20 g/l peptide digest of animal tissue, 2 g/l dextrose, 2g/l sodium chloride, 0,4 g/l disodium phosphate and 2,5 g sodium carbonate. 18,5 g of TH-broth powder was dissolved into 0,5 litre of distilled water. 20% Glycerol broth was made by adding 3 g sodium chloride, 3 g Tryptone, 1,5 g yeast extract and 60 ml glycerol (20% of total amount) into 240 ml distilled water. All broths were autoclaved before used.

One strain of *Staphylococcus aureus* (1685-4) were obtained from a previous experiment done in our group (Lewandowska-Sabat et al., 2013), and collected from a 20% glycerol stock stored at -20 C°. The culture was plated on blood agar plates, and incubated in 37 C° for 24 hours to multiply the number of bacteria. After incubation, some of the colonies lacked hemolysis in the

blood agar, so a colony with classic phenotypic appearance including hemolysis were collected and added to 5 ml LB-broth. Another classic phenotype colony was sowed into a new blood agar plate to verify that the culture was pure. Further the 5ml LB-broth with the bacteria were shaken in incubation for 24 hours at 37 C°, and 0,5 ml of the media were transferred into 30 ml of new LB-broth. The culture was shaken in incubation for another 24 hours at 37 C°, before measuring growth by optical density (OD) at 600 nm to ensure that the culture was in mid-log-phase.

Another batch of *Staphylococcus aureus* (1685-4) were obtained on a blood agar plate directly from the original strain at The Norwegian Veterinary institute. All colonies from this batch had phenotypical hemolysis on blood agar. One colony was transferred into 5ml LB-broth, and shaken in incubation for 24 hours at 37 C°. 0,5 ml of the media were transferred into 30 ml of new LB-broth, and shaken in incubation for another 24 hours at 37 C°. Growth was measured by optical density (OD) at 600 nm to ensure that the culture was in mid-log-phase.

Two strains of *S. agalactiae* (ST 103 and ST 12) were obtained from The Norwegian Veterinary Institute; ST 103 number 2013-01-MB-235-27, and ST 12 number 2014-01-MB-48, and were chosen based on results from research by The Norwegian Veterinary Institute.(Jorgensen et al., 2016). These bovine adapted strains were originally isolated from milk samples, and a colony was collected from each blood agar plate and added into 5 ml TH-broth separately. The cultures were further shaken in incubation for 24 hours at 37 C°, and 0,5 ml of the media were transferred into two bottles with 30 ml new TH-broth. The cultures were shaken in incubation for another 24 hours at 37 C°, before measuring growth by optical density (OD) at 600 nm to ensure that the culture was in mid-log-phase.

The *S. aureus* and *S. agalactiae* cultures were further aliquoted and frozen in 20% glycerol broth at -80 C°. The final number of colony-forming units (CFU) was determined by serial dilutions and plating on blood agar plates. When the final number in each batch was determined, the amount of medium with bacteria needed for infecting cells in a multiplicity of infection of 1 (MOI, 1 bacterium pr cell, on average) was calculated. Bacteria in this study all came from

aliquots of the same batch, and were diluted 1:10 before infecting cells.

Bacterial infection with *Streptococcus agalactiae*

The wells with early-stage macrophages were divided into four classes with as equal number of wells and cells per class as possible. Two classes were infected with ST103 and ST12 in a multiplicity of infection (MOI) of 1, by adding 1,5 million bacteria per 1,5 million cells.
In the third cell class (positive control) 1 mg/ml of lipopolysaccharides (LPS, rough strains) from Salmonella Minnesota Re 595 (re mutant Sigma Aldrich) was added, and the last cell class was left uninfected (negative control). After 1 hour of exposure in 37 C°, 1 % penicillin/streptomycin (60 pg/mL penicillin and 100 μg/ml streptomycin) were added to each well to prevent growth of remaining extracellular bacteria, and the controls and the infected cells were treated equally. Inhibition of bacterial growth by antibiotics was verified by microscopy. Incubation was continued for one additional hour for the cells exposed to LPS, and for 5 more hours (total of 6 hours) for cells infected with bacteria and the negative control cell classes. After incubation, media was aspirated and the cells were collected using cell scraper and the wells were controlled for remaining cells with microscopy. The cells were centrifuged (400xg, 5 min at -4C°.), and the pellet was washed with cold PBS buffer, snap frozen in liquid nitrogen and stored at -80 C°.

Bacterial infection with *Staphylococcus aureus*

The same method for bacterial infection as for the cells infected with *S. agalactiae* was applied to macrophages infected with *S. aureus* with a MOI of 1, by adding 1,5 million bacteria per 1,5 million cells. After 1 hour of exposure in 37C°, the cells were observed to be dead. The protocol was repeated with a MOI of 0,1 and with the original bacterial strain with no change in result. No cells could be collected for further procedure and were eliminated from the experiment.

RNA Isolation

24 RNA samples from six animals were used (i.e. 6 samples form ST103 infected macrophages, 6 samples from ST12 infected macrophages, 6 samples from LPS exposed macrophages (positive control) and 6 samples from uninfected macrophages (negative control)). Total RNA was isolated from all four classes with the infected cells, positive and negative control using the

MirVANA isolation kit (Ambion, Austin, TX), following the manufacturer´s instructions. The cells were disrupted in 600 µl Lysis/Binding solution per sample and vortexed for homogenization. 60 µl of miRNA Homogenate Additive were added to the cell lysate, and vortexed. Further the mixture was left on ice for 10 minutes, before 600 µl Acid:Phenol:Chloroform was added to each sample. The samples were vortexed and centrifuged for 5 minutes at 10,000xg at room temperature, before the aqueous phase was transferred to a new tube. 750 µl room temperature 100% ethanol was added to the aqueous phase tube, and the mixture was then pipetted onto a Filter Cartridge placed in a collection tube. The tube was centrifuged for 15 sec at 10,000xg, and the flow-through was removed. The Filter Cartridge were washed with 3 different washing solutions, before 100 µl pre-heated nuclease-free water was added to eluate the RNA from the filter. The elution was repeated to make sure all RNA was collected, before the eluted RNA was stored at -80C°.

The concentration and quality of the isolated RNA was measured using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, USA) and 2100 BioAnalyzer (Agilent RNA 600 Nano, Agilent Technologies, Palo Alto, USA), respectively. The RNA integrity numbers (RIN), concentrations and OD A260/280 are listed in **Table 1.**

Table 1: The RNA integrity numbers (RIN), concentrations and OD A260/280. The OD ratio of RNA is generally accepted as pure with a ratio of ~2.0 (ThermoScientific), and the quality is measured by RIN between 1 and 10, with 1 being the most degraded profile and 10 being the most intact (Mueller, Lightfoot, & Schroeder, 2004).:

Sample Name	Concentration (ng/µl)	OD A260/280	RIN
2_control	110.9	2.0	8.5
2_LPS	88.7	1.8	8.6
2_ST12	84.2	2.0	7.6
2_ST103	72.2	1.8	8.7
3_control	107.2	2.0	7.8
3_LPS	93.4	2.0	7.5
3_ST12	95.7	2.0	7.0
3_ST103	68.5	1.9	6.6
4_control	78.1	2.4	6.8
4_LPS	37.9	3.4	6.3
4_ST12	118.7	2.4	7.8
4_ST103	77.9	2.4	6.8
5_control	72.4	2.5	8.1
5_LPS	52.9	3.8	7.9
5_ST12	164.2	2.1	7.1
5_ST103	87.9	2.5	8.2
8_control	57.8	2.0	7.7
8_LPS	83.6	2.0	6.7
8_ST12	53.6	2.0	N/A
8_ST103	124.5	1.9	7.1
9_control	110.1	2.0	8.2
9_LPS	89.6	2.1	7.4
9_ST12	86.6	2.0	N/A
9_ST103	57.3	2.1	7.3

All RNA samples were treated with amplification grade DNase I (Invitrogen) to remove any traces of genomic DNA. 1 µg RNA sample, 1µl 10X DNase I Reaction Buffer and 1 µl DNase I Amp Grade 1 U/µl was mixed in a RNase-free tube on ice and added DEPC-treated water to a total volume of 10µl. The mixture was further incubated in room temperature for 15 minutes, before inactivating the DNase I by adding 1 µl of 25 mM EDTA solution, and heating the sample for 10 minutes at 65 C°.

cDNA synthesis

A total of 200 ng RNA was used for cDNA synthesis, using "Tetro cDNA synthesis kit" (Nordic BioSite, Norway). A premix for the cDNA synthesis were made for each RNA sample; by mixing 200 ng RNA, 1 µl Oligo $(dT)_{18}$ primer, 1 µl 10mM dNTP mix, 1 µl RiboSafe RNAse inhibitor, 1 µl Tetro Reverse Transcritpase (200u/µl) with 4 µl 5xRT-buffer, and the remaining volume with distilled H_2O to get a total volume of 20 µl. The cDNA synthesis was done by incubating the premix for 30 minutes at 45 C°, before terminating the reaction with incubating for 5 minutes at 85 C°.

Reverse transcription – quantitative PCR

cDNA equivalent to 5 ng of total RNA was used in qPCR reactions by diluting the cDNA. Each sample was set up in triplicate using "Express SYBR® GreenER™ qPCR SuperMix Universal" with premixed ROX (Invitrogen) according to the manufacturer´s recommendations using 20 µl reaction volumes. The samples were prepared for qPCR by mixing 10 µl SYBR, 0,8 µl 10uMPrimer F/R, 5 µl (5 ng) cDNA and 4,2 µl distilled H_2O; one mix for each primer (a total of 7 premixes for each cDNA sample).

Transcript levels were analysed using a 7900HT Fast Real Time PCR System (Applied Biosystems) and the standard program: 50 C° for 2 minutes, 95 C° for 2 minutes, 40 cycles of 95 C° for 15 seconds and 60 C° for 1 minute, followed by melting curve analyses.

Gene-specific primers were either derived from literature (TNFα: (Lewandowska-Sabat et al., 2013)) or designed using Primer3 ver.0.4.0. The genes analysed are: *TNF-α, IL-1β, IL-6, IL-8, IL-10* and *TGFβ1*. The primer sequences are listed in **Table 2.**

The *peptidylproplyl isomerase A (PPIA)* housekeeping gene were used in the current study as the reference gene, as it has been shown to be one of the most stable genes for gene expression studies in cattle macrophages (Lewandowska-Sabat et al., 2013), lymphocytes (Spalenza et al., 2011), and in human LPS-stimulated monocytes (Piehler, Grimholt, Ovstebo, & Berg, 2010). In the experiment, PPIA was expressed at the same level in the cells stimulated with the bacterial strains and LPS, as in the negative controls. Negative controls with no added template were included for all primer pairs (no template control: NTP), and each RNA sample underwent no RT control reactions, and each primer were run in qPCR to check for genomic DNA contamination (no RT control).

The efficiencies of all primer pairs were tested by template dilution series using pooled cDNA from control and infected cells and were 100% (+/-10).

Table 2: The primer sequences:

Gene symbol, accession no.	Primers (5'→3')	Amplicon (bp)
TNFα, NM_173966.3 *tumor necrosis factor alpha*	TCTTCTCAAGCCTCAAGTAACAAG CCATGAGGGCATTGGCATAC	103
IL1β, NM_174093.1 *interleukin-1 beta*	AAAAATCCCTGGTGCTGGCT CATGCAGAACACCACTTCTCG	89
IL-6, NM_173923.2 *interleukin-6*	CCTGAAGCAAAAGATCGCAGA TGCGTTCTTTACCCACTCGT	97
IL-8, NM_173925.2 *interleukin-8*	AACGAGGTCTGCCTAAACCC TGCTTCTCAGCTCTCTTCACAA	77
IL-10, NM_174088.1 *interleukin-10*	TATCCACTTGCCAACCAGCC GGCAACCCAGGTAACCCTTA	152
TGFβ1, NM_001166068.1 *Transforming growth factor beta 1*	CAATTCCTGGCGCTACCTCA GCCCTCTATTTCCTCTCTGCG	121

Initial analyses of the RT-qPCR data were performed using RQ-manager 1.2 (Applied Biosystems). Standard deviation of ≤0.3 per triplicate was accepted.

The ΔCt method (**Table 3)** was used to calculate RT-qPCR data, and normalized gene expression was calculated. Fold change was calculated relative to the negative, unexposed control. Reciprocal values of fold change were used for down regulated gene expression (ie TGFβ1) to facilitate the interpretation of the results (se **table 4**).

Table 3: Calculating method for RT qPCR data

$\Delta Ct_{treated}$ = Ct Target Gene $X_{treated}$ – Ct Reference Gene $Y_{treated}$
$\Delta Ct_{control}$ = Ct Target Gene $X_{control}$ – Ct Reference Gene $Y_{control}$
$\Delta\Delta Ct = \Delta Ct_{target\ gene} - \Delta Ct_{reference\ gene}$
Normalized gene expression level = $2^{-\Delta\Delta Ct}$

The differences of normalized gene expression levels between control and infected cells for each gene were tested using "Wilcoxon matched pairs signed rank test" using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The pairwise differences between responses to LPS, ST103 and ST12 were tested using log-transformed fold change values for each treatment and gene by RM one-way ANOVA and Tukey´s multiple comparison test using GraphPad Prism. The significance level was determined at P ≤0.05

Results

Infection with *S. agalactiae* (ST103 and ST12)

We investigated the early phase response of bovine monocyte-derived macrophages infected *in vitro* with two different sequence types of live *S. agalactiae* (ST103 and ST12), monocyte-derived macrophages stimulated by LPS and compared to a negative control; by examining the transcription level of six macrophage-associated cytokines with RT-qPCR.

The calculated normalized gene expression levels are shown in **Table 4 and 5.**

$2^{-\Delta Ct}$ values shows the gene expression of the cytokines within the groups of stimulated monocyte-derived macrophages (ST12, ST103 and LPS) by comparing the Ct-values with the reference gene. The $2^{-\Delta\Delta Ct}$ values shows the normalized gene expression of the cytokines in the stimulated monocyte-derived macrophages compared to the uninfected negative control, and thus indicate the comparison of the response for each cytokine between the groups of stimulated monocyte-derived macrophages.

Table 4 - Gene expression levels

		TNFα		IL-1β		IL-6	
Animal	**Type**	$2^{-\Delta Ct}$	$2^{-\Delta\Delta Ct}$	$2^{-\Delta Ct}$	$2^{-\Delta\Delta Ct}$	$2^{-\Delta Ct}$	$2^{-\Delta\Delta Ct}$
2	**Control**	0,006077396		1,616939463		0,002925614	
3	**Control**	0,001893484		0,258482796		0,000322985	
4	**Control**	0,003187671		0,445936955		0,000700349	
5	**Control**	0,007664675		1,182196892		0,031033294	
8	**Control**	0,00628986		1,336972076		0,002183336	
9	**Control**	0,003082445		1,050728061		0,021622962	
2	**LPS**	0,224704408	36,97379779	46,83560079	28,96558706	0,446878952	152,7470457
3	**LPS**	0,037112646	19,60019361	2,613532923	10,11105173	0,007078043	21,91446672
4	**LPS**	0,017400356	5,458641688	3,079486919	6,90565535	0,156998898	224,1724484
5	**LPS**	0,019952034	2,603115514	3,423465167	2,89585025	0,946607222	30,50295627
8	**LPS**	0,038152542	6,065721809	10,31686925	7,71659292	0,135908633	62,24816196
9	**LPS**	0,023014842	7,46642432	7,540080886	7,176053602	0,093404681	4,319698637
2	**ST12**	0,367356065	60,44629465	33,56507591	20,75839985	0,144144897	49,26995788
3	**ST12**	0,208998187	110,3776042	5,558926602	21,50598294	0,003825563	11,84439825
4	**ST12**	0,019179926	6,016908148	0,774505649	1,73680526	0,040675301	58,07863601
5	**ST12**	0,073878873	9,638878748	2,020859703	1,709410434	0,333507012	10,74674857
8	**ST12**	0,053940374	8,575766749	1,455953554	1,088993241	0,020875137	9,561121368
9	**ST12**	0,053980407	17,51220488	2,208298708	2,101684338	0,09180696	4,245808591
2	**ST103**	0,239212939	39,36109179	28,54579493	17,65421377	0,222300998	75,98438152
3	**ST103**	0,12071847	63,75469427	4,204630433	16,2665775	0,002151843	6,662363177
4	**ST103**	0,037209651	11,67298802	2,782491602	6,239652423	0,009177484	13,10416177
5	**ST103**	0,07103271	9,267543617	2,870356636	2,427985266	0,252678707	8,142181229
8	**ST103**	0,015022131	2,388309268	6,674954488	4,992590802	0,007935209	3,634443136
9	**ST103**	0,027450559	8,905449915	3,488082912	3,319681887	0,028684066	1,326555774

Table 5 - Gene expression levels continued

		IL-8		IL-10		TGF-β		TGF-β (-1)
Animal	**Type**	$2^{-\Delta Ct}$	$2^{-\Delta\Delta Ct}$	$2^{-\Delta Ct}$	$2^{-\Delta\Delta Ct}$	$2^{-\Delta Ct}$	$2^{-\Delta\Delta Ct}$	$2^{-\Delta\Delta Ct}$
2	**Control**	0,654258046		0,21642329		0,045585348		
3	**Control**	0,619366246		0,009204459		0,203656204		
4	**Control**	0,466144128		0,003625055		0,064836559		
5	**Control**	0,245547607		0,001422337		0,167073404		
8	**Control**	0,737337822		0,011420258		0,095311053		
9	**Control**	1,516376146		0,007773216		0,017381246		
2	**LPS**	6,517230406	9,961253737	3,380926532	15,62182393	0,057540677	1,262262531	1,262262531
3	**LPS**	1,510146312	2,43821216	0,128011471	13,90754948	0,435349609	2,137669275	2,137669275
4	**LPS**	7,638404563	16,38635799	0,01199507	3,308934346	0,042318912	0,652701382	-1,532094196
5	**LPS**	0,622847553	2,536565357	0,002792161	1,963079138	0,052982498	0,317121076	-3,153369727
8	**LPS**	13,53242043	18,35308055	0,05127905	4,490183278	0,045544948	0,477855888	-2,092681131
9	**LPS**	4,601743807	3,034698099	0,031779106	4,088282812	0,129723012	7,463389606	7,463389606
2	**ST12**	9,454682425	14,45099909	21,31329386	98,47966848	0,035045929	0,768798095	-1,300731631
3	**ST12**	5,886551315	9,504152597	0,475290015	51,63693031	0,150200376	0,737519279	-1,35589676
4	**ST12**	5,174353671	11,10033004	0,076019376	20,97054329	0,011533985	0,177893228	-5,62134944
5	**ST12**	1,432724282	5,834812651	0,033394832	23,47884173	0,043357889	0,259514011	-3,853356503
8	**ST12**	7,13361324	9,674823441	0,034996772	3,064446816	0,013197163	0,138464144	-7,222086334
9	**ST12**	10,06219396	6,635684683	0,122838582	15,80280039	0,005382985	0,309700725	-3,228923662
2	**ST103**	5,185575106	7,92588664	9,152348579	42,28911118	0,037654898	0,826030715	-1,210608736
3	**ST103**	5,91145117	9,544354748	0,162350305	17,63822322	0,115771978	0,56846772	-1,759114837
4	**ST103**	5,04328702	10,81915809	0,080462535	22,19622355	0,034133599	0,52645605	-1,899493796
5	**ST103**	0,828462136	3,373936918	0,012937064	9,095636925	0,078328474	0,468826709	-2,13298428
8	**ST103**	12,39912722	16,81607379	0,054391386	4,76271097	0,031520648	0,330713459	-3,023765655
9	**ST103**	4,646950681	3,06451054	0,099126871	12,75236273	0,036200966	2,082760089	2,082760089

The normalized gene expression levels show that *TNF-α*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* were significantly up-regulated by ST12, ST103 and LPS compared to the negative control ($P \leq 0.05$). *TGFβ1* were significantly down-regulated only in the macrophages infected with ST12 ($P \leq 0.05$). *IL-6* and *IL-10* displayed significantly different responses both between ST103 and LPS, and between ST12 and LPS. ($P \leq 0.05$). When comparing the transcription levels of the cytokines between the two strains of *S. agalactiae*, we did not observe significantly different expression of any of the six cytokines. (**Figure 1).**

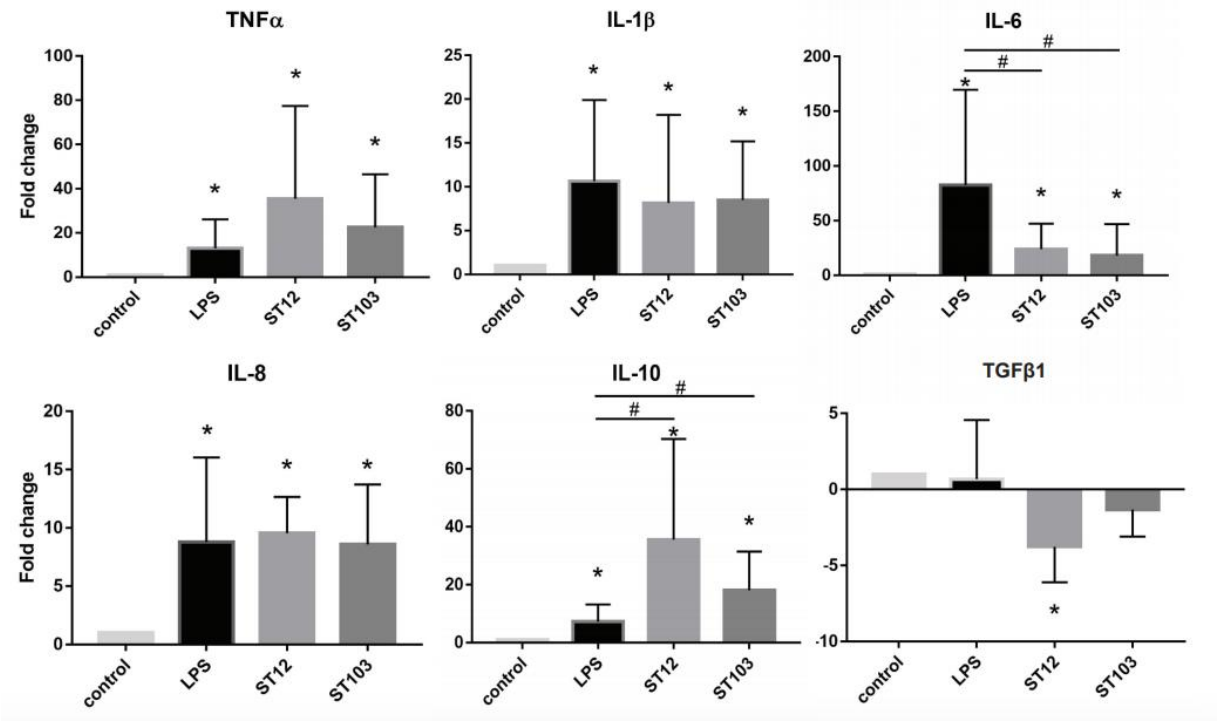

*Figure 1 - mRNA gene expression levels, presented as fold change relative to negative control, and as mean values with SD. The significant difference in expression between the ST103 and ST12 infected cells, and LPS stimulated cells, respectively, compared to uninfected controls are presented with * (P ≤ 0.05). The significant differences between responses to LPS vs ST103, and LPS vs ST12, respectively, are denoted with # (P ≤ 0.05).*

Infection with *S. aureus*

We originally planned to compare the early phase response from the cells infected with ST12 and ST103 against the immune response of monocyte-derived macrophages infected with *Staphylococcus aureus in vitro.* After infecting the macrophages with *S. aureus* with a MOI of 1, and incubating the cells for 1 hour of exposure in 37 C°, we observed that the majority of cells were dead. The experiment was repeated with a lower MOI (0,1 bacteria per cell) with no change in result, indicating that it was not the number of bacteria per cell or a mistake in the dilution series that caused the cell lysis.

When troubleshooting the method, the most noticeable aberration was the mixed bacterial culture on the blood agar plates, where some colonies lacked the zone of hemolysis. There was only one Eppendorf tube left with bacteria stored at -20 C° from the previous study to multiply, and the classical phenotype of *S. aureus* with hemolysis were collected for the subsequent infection in

this experiment. In the method from the previous study the cultivation was done by scraping all the colonies from the original blood agar plate (obtained from the Veterinary Institute) straight into LB-broth without investigating the phenotype in advance. Hence, there was a possibility that the batch already on that point of time was mixed (with both hemolytic and non-hemolytic cultures). However, since this was the last Eppendorf tube left with bacteria from the previous study, it would be impossible to say whether the mixed culture was a result of direct cultivation in LB-broth, or if the mixed strain had been contaminated during storage.

Thus, we collected the original strain of *Staphylococcus aureus* (1685-4) from The Norwegian Veterinary institute and repeated the protocol (i.e. one colony from the blood agar was collected and cultivated in LB-broth) to verify that the old batch obtained the same virulence behaviour as the original strain. When infecting the macrophages with this new batch, the cells still died and dissolved. The macrophages showed signs of stress after few minutes with the bacteria **(Figure 2-4).** The cells infected with *S. aureus* were therefore eliminated from the experiment.

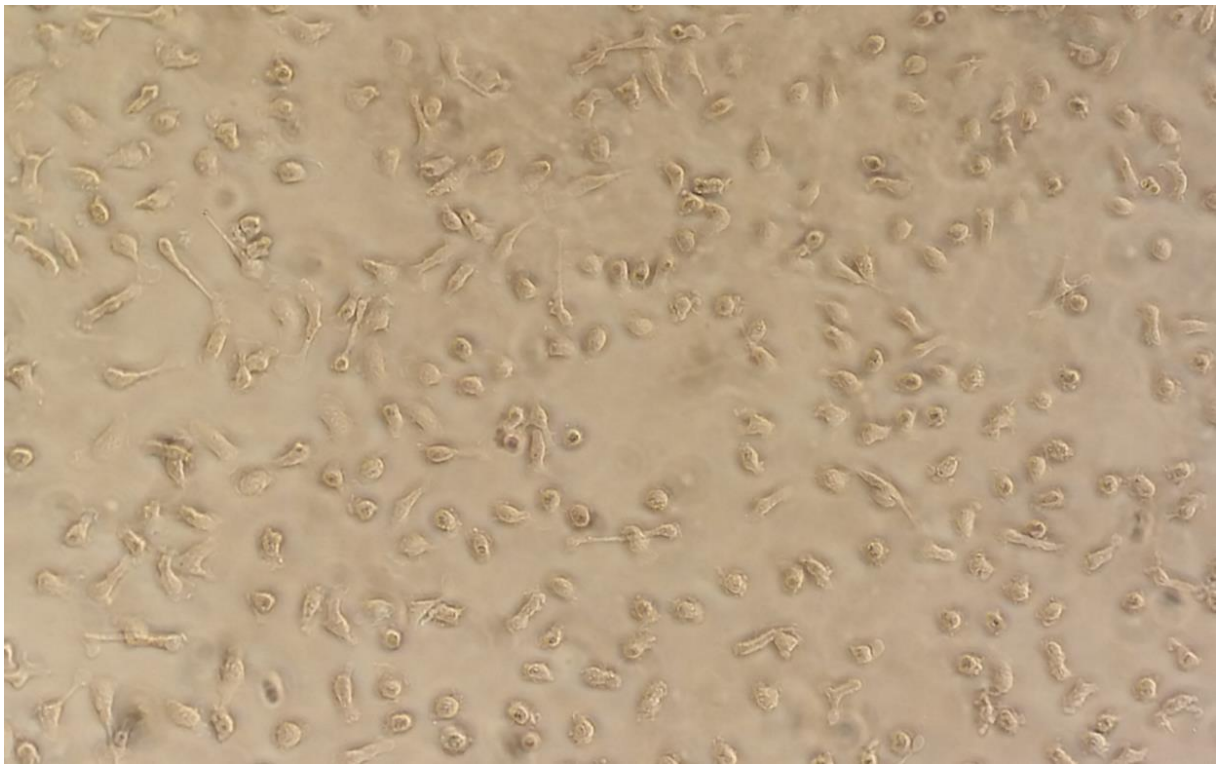

Figure 2. Bovine monocyte-derived macrophages at infection, phase contrast microscopy 10x. The cells are phenotypic macrophages, with elongated, adherent cells with a distinct nucleus.

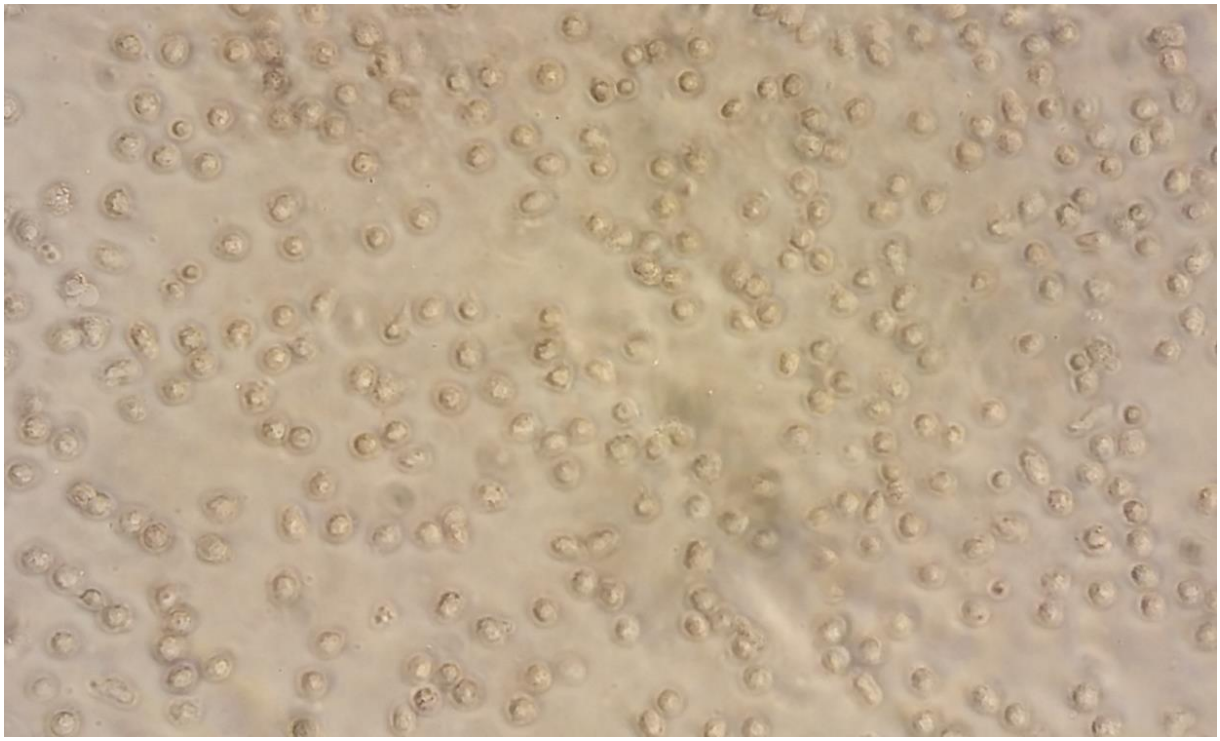

Figure 3. Bovine monocyte-derived macrophages, 10 minutes post infection with S.aureus in vitro, phase contrast microscopy 10x. The cells are more strained, circular and the nucleus is not as marked as earlier.

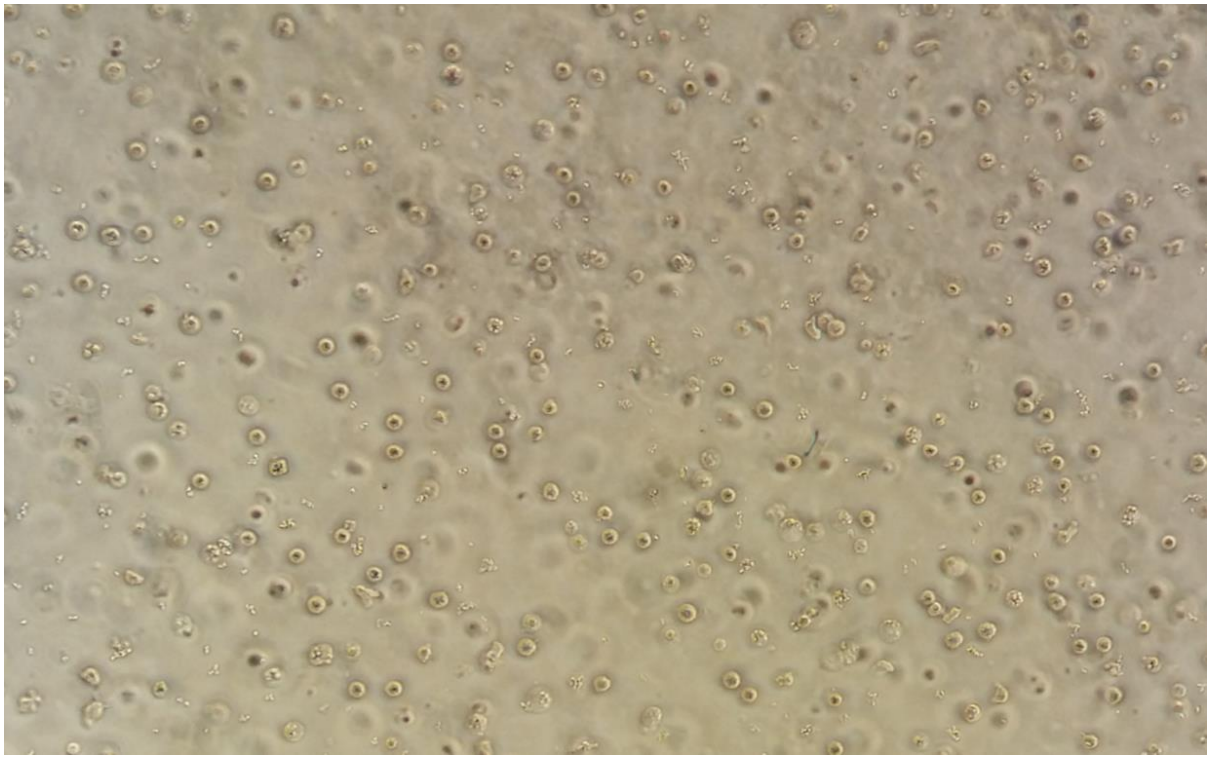

Figure 4. Bovine monocyte-derived macrophages, 1 hour post infection with S. aureus in vitro, phase contrast microscope 10x. The cells are lysed and dissolved, there is cell debris in the medium and there are no viable macrophages left.

Discussion

The aim of this project was to investigate the immune response when bovine monocyte-derived macrophages was infected with two strains of *S. agalactiae in vitro*, and to compare this response against the response against *S. aureus.* The project had particular emphasis on examining the transcription level of six macrophage-associated cytokines, and whether the response against *S. agalactiae* indicated a classical or alternative activation of macrophages.

When we infected the macrophages with *S. aureus,* we noticed that the cells died after short time of exposure with the bacteria which could indicate that there are pre-formed toxins that kills the cells. As mentioned earlier, the only known aberration in our experiment was the lacking of hemolysis zone in some of the bacterial cultures, where we sampled one phenotypical colony for subsequent cultivating and infection. Hence, we tried to infect the cells with a lower MOI, and with the original strain with no change in result. This indicates that the bacteria in the previous study by our group did not behave as the original strain, since the method for infection were equal, and the original strain also managed to lyse the cells after short time of exposure. One theory of this could be that the bacterial culture during storage or cultivation directly in LB-broth down-regulated some of the virulence factors, including haemolysins (or that a positive selection for bacteria missing haemolysins occurred), and that some of the bacteria in the first batch consequently were less virulent. This could explain why the cells survived in the last study but not this time. There are many studies indicating that virulence factors like for instance α-haemolysine are leukocidic, and that *S. aureus* are able to kill macrophages by forming pores in the cell wall (Flannagan, Heit, & Heinrichs, 2015; Menestrina, 1986; Thammavongsa, Kim, Missiakas, & Schneewind, 2015). This could support the theory of the cells being able to survive with this virulence factor absent in the previous study, and by that managed to engulf the bacteria and still produce an inflammatory response.

Hence no cells in our experiment could be collected for further investigation, and the experiment was taken out of this project, and the aim was reduced to compare and investigate the transcription level of the cytokines in the macrophages infected with the two strains of *S. agalactiae in vitro.*

There is dim knowledge of the immune response in cattle and macrophages against *S. agalactiae*. Some studies performed on mice *in vivo* indicates high levels of *TNF-α* and *IL-1β* when macrophages responded to Group B Streptococcus. (Rosati et al., 1998). It is also seen that *S. agalactiae* induces the production of *TNF-α* in human mononuclear cells *in vivo*. (Williams et al., 1993). A mouse model where mice were infected with a strain of *S. agalactiae* isolated from bovine mastitis showed an increase in *TNF-α* , *IL-1β* and *IL-6* from resident macrophages in the mammary gland. After 72 hours, they observed a decrease in these cytokines, with a correlated increase in *IL-12* and *IL-10*. (Trigo et al., 2009). Studies of the molecular and virulence characteristics of different bovine *S. agalactiae* isolates showed that ST103 displayed a significantly higher hemolytic activity and cytotoxicity compared to most of the other bovine strains, and that ST103 also was associated with biofilm formation, growth ability in milk and adhesion to bovine mammary epithelial cells. (Pang et al., 2017). On the contrary, characterization of ST12 showed that this strain display great resistance to erythromycin and clindamycin, and have the ability to survive within human macrophages (Jiang et al., 2016; Korir et al., 2017). Thus, it was interesting to investigate if the bovine macrophages would respond in a similar way as the murine and human macrophages when infected with *S. agalactiae*, and if the cytokine response would differ between the strains based on the difference in characteristics. In our study, no significant expression was found when comparing the cytokine response between ST103 and ST12 *in vitro*, however there was some significant differences when comparing the response against the uninfected cells, and against the cells activated by LPS. We found that *TNF-α*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* were up-regulated compared to the negative control, indicating that the macrophages have activation of both pro-inflammatory and anti-inflammatory cytokines when infected with *S. agalactiae,* and that the bovine macrophages respond in a similar way against the pathogen as humane and murine macrophages. *TGFβ1* were significantly down-regulated only in the macrophages infected with ST12, indicating that there might be a difference in the cytokine response between macrophages infected with ST12 vs ST103. However, as no significant difference between the two strains was found, we could not draw a conclusion based on this result. As the two strains are members of the same bacteria, it is likely to think that the PAMPs recognized by macrophages and the rest of the innate immune system might be too similar to cause a difference in the response.

There is evidence that pathogens may use subversion of macrophage activation into an alternative pathway as a strategy to escape the immune response and to survive. (Muraille, Leo, & Moser, 2014). In studies on mice, it is seen that *S. agalactiae* can produce glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a virulence factor that induces production of *IL*-10 early in the immune response, which further impairs neutrophil recruitment and by that preventing the bacterial clearance. (Madureira et al., 2011; Seifert, McArthur, Bleiweis, & Brady, 2003). Nevertheless, other studies suggest that *S. agalactiae* may induce the classical activation pathway in human monocyte-derived and placental macrophages (Sutton, Rogers, Doster, Gaddy, & Aronoff, 2017), and so this needs more investigation. LPS is known to activate macrophages in the classical pathway (Mantovani et al., 2004), and this is consistent with our results from the LPS stimulated macrophages. Further we found that *IL-6* displayed a lower response both between ST103 and LPS, and between ST12 and LPS, which indicates that the activation of IL-6 is less prominent when infected with *S. agalactiae* than in the macrophages stimulated by LPS. We also found that *IL-10* displayed a higher response between ST12 and LPS, and between ST103 and LPS, indicating that the activation of IL-10 is elevated when the macrophages are infected with *S. agalactiae* compared to the cells stimulated with LPS. Hence, this high level of IL-10 (anti-inflammatory cytokine) and low level of IL-6 (pro-inflammatory cytokine) suggest that the macrophages infected with *S. agalactiae* might be activated in the alternative pathway compared to the LPS-stimulated cells with the known classical pathway. This supports our hypothesis that *S. agalactiae* causing predominantly subclinical mastitis would show evidence of alternative macrophage activation, and that this could be a mechanism the bacteria activate to avoid the immune response. However, the results are limited as this was a small study with few animals, and requires further investigation with a larger sample size. Based on these methods and results, our group pursued this project and investigated the miRNA repertoire in the isolated RNA. (Lewandowska-Sabat et al., 2018). This is elaborated in the article in **Appendix 1**. There we found that the ST12 strain induces miRNA expression associated with a stronger inflammatory response than the ST103 strain, and that both strains in terms of miRNA expression seem to activate the macrophages away from the classical activation. These results also support our hypothesis that infection with *S. agalactiae* lead to polarization of the macrophages toward the alternative activation. It should be noted, that our results are based on *in vitro* studies of blood monocyte-derived macrophages, and the hypothesis

also needs to be evaluated with macrophages derived from bovine mammary glands infected by *S. agalactiae*.

In conclusion, we successfully isolated bovine monocyte-derived macrophages and infected the cells with two different strains of *Streptococcus agalactiae* (ST12 and ST103). The transcript levels of both pro-inflammatory and anti-inflammatory cytokines was investigated, and our results point in the direction of a polarization towards the alternative activation when the macrophages are infected with *S. agalactiae* compared to macrophages stimulated by LPS. This is supported by the results in the additional study on miRNA expression. Thus, our findings suggest that the alternative activation of macrophages might be an escape mechanism for *S. agalactiae* to escape the immune system during an infection. Taken together, this area of knowledge needs further investigation to obtain a better understanding of the early immune response, and the polarization in bovine macrophages infected with *S. agalactiae* during mastitis in cattle.

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MicroRNA expression profiles of bovine monocyte-derived macrophages infected in vitro with two strains of *Streptococcus agalactiae*

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Abstract

Background: MicroRNAs (miRNAs) are short, non-coding RNAs that regulate gene expression at the post-transcriptional level and play a key role in the control of innate and adaptive immune responses. For a subclinical infection such as bovine streptococcal mastitis, early detection is a great challenge, and miRNA profiling could potentially assist in the diagnosis and contribute to the understanding of the pathogenicity and defense mechanisms. We have examined the miRNA repertoire and the transcript level of six key immune genes [*tumor necrosis factor alpha* (*TNFα*), *interleukin-1 beta* (*IL-1β*), *interleukin-6* (*IL-6*), *interleukin-8* (*IL-8*), *interleukin-10* (*IL-10*) and *transforming growth factor beta 1* (*TGFβ1*)] during the early phase response of bovine immature macrophages to in vitro infection with live *Streptococcus agalactiae*. Next generation sequencing of small RNA libraries from 20 cultures of blood monocyte-derived macrophages exposed to either one of two sequence types of *S. agalactiae* (ST103 or ST12) for 6 h in vitro and unchallenged controls was performed.

Results: Analyzes of over 356 million high quality sequence reads, revealed differential expression of 17 and 44 miRNAs ($P < 0.05$) in macrophages infected with ST103 and ST12, respectively, versus unchallenged control cultures. We also identified the expression of 31 potentially novel bovine miRNAs. Pathway analysis of the differentially regulated miRNAs and their predicted target genes in the macrophages infected with ST12 revealed significant enrichment for inflammatory response and apoptosis, while significant enrichment for integrin and GABA signaling were found in ST103 infected macrophages. Furthermore, both bacterial strains regulated miRNAs involved in the alternative activation of macrophages. The transcript levels of *TNF-α*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* were significantly up-regulated by both bacterial strains, however the expression of *TGFβ1* was significantly down-regulated only by ST12.

Conclusions: Our study identified pathogen-induced differential regulation of miRNAs controlling inflammation and polarization in bovine macrophages. This implies that miRNAs have potential to serve as biomarkers for early detection of bacterial infection.

Keywords: Cattle, qPCR, Macrophages, Microrna sequencing, *Streptococcus agalactiae*, Subclinical mastitis

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Background

MicroRNAs (miRNAs) are an abundant class of non-coding, small RNA molecules (19–24 nt long), which bind to the 3'UTR of target mRNAs to repress the translation into protein or accelerate the decay of expressed transcripts (affecting 60% of all mRNA transcripts [1]). Microorganisms are known to alter the host transcriptome and proteome in several ways, affecting miRNA is one of several such mechanisms. For example, miRNA induced by *herpesviridae* and *Hepatitis C* virus appear to work as an escape mechanism by dampening the host's immune system [2] and miRNA induced by the latter is currently being targeted for therapy [3]. Up-regulation of miRNA will mostly attenuate the immune response, but it is unclear if this should be understood as a pathogen escape mechanism or a survival mechanism on the part of the host to avoid immunopathology.

Only a few studies describe miRNA regulation in the context of bovine bacterial mastitis [4]. Modification of miRNA expression were reported in response to *Streptococcus uberis* infection of primary bovine mammary epithelial cells (BMEs) and circulating monocytes from blood and milk [5, 6]. In addition, profiling of the complete miRNA content (miRNome) of BMEs infected with *Staphylococcus aureus* and *Escherichia coli* [7], and milk exosomes from *S. aureus* infected cows [8], revealed several pathogen directed miRNAs with enriched role in immunity, infection and cellular processes. MiRNAs have also gained prominence as potential biomarkers for a range of infections and diseases, and more recent studies profiling serum miRNAs from a bovine *Mycobacterium avium subsp. paratuberculosis* infection model demonstrated high stability of circulating miRNAs [9].

Macrophages are critical effectors and regulators of inflammation serving as the first line of defense against invading pathogens. Intramammary infections will activate macrophages to produce pro-inflammatory cytokines [e.g. *tumor necrosis factor alpha* (*TNFα*), *interleukin-1 beta* (*IL-1β*), *interleukin-6* (*IL-6*) and *interleukin-8* (*IL-8*)] required to kill intracellular pathogens. However, the balance between pro- and anti-inflammatory signals [e.g. *interleukin-10* (*IL-10*) and *transforming growth factor beta 1* (*TGFβ1*)] is crucial for immune regulation of the inflammation and preventing chronic conditions. The presence of microbes together with the host microenvironment underlies the diversity of activation of macrophages [10], and in the bovine mammary gland, the status of differential macrophage activation may be pivotal for the defense and resolution of mastitis [11].

In this study, we examined the expression of selected genes and the miRNA profiles of immature macrophages (primary cells) infected in vitro with the ST103 or ST12 strain of *Streptococcus agalactiae*, a leading causative agent of subclinical bovine mastitis. ST103 is prevalent in farms with considerable environmental contamination, while ST12, a strain usually associated with colonization of pregnant women, has been found in cattle herds with no positive environmental samples [12, 13]. To the best of our knowledge, this study represents the first report of next generation sequencing used to profile the host miRNAs response to *S. agalactiae*. Furthermore, the identification of differentially expressed miRNAs in infected immature macrophages may provide a basis for development of biomarker assays for early detection of subclinical mastitis.

Methods

Animals and cell isolation

Six healthy Norwegian Red (NR) cows aged 4–7 years were used for miRNAs sequencing, and six healthy NR cows aged 2.5–7 years were used for reverse transcription-quantitative PCR (RT-qPCR). Five of the cows were included in both experiments (for details see Additional file 1: Table S1 and Additional file 2: Figure S1). All animals were maintained under uniform housing conditions and nutritional regimens at the Norwegian University of Life Sciences (NMBU) herd. Blood sampling was performed by certified personnel and conducted in agreement with the provisions enforced by the Norwegian Animal Research Authority. Five hundred ml of blood was collected from the neck of each animal in sterile glass bottles with sodium citrate as anticoagulant. Peripheral blood mononuclear cells (PBMC) were extracted by density gradient centrifugation (2210×g, 30 min) on lymphoprep (Axis-Shield, Norway). CD14+ cells were extracted by positive selection of monocyte differentiation antigen CD14 using anti-human CD14 MACS MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions and as described earlier by Lewandowska-Sabat et al. [14]. Purity of selected cells was verified by flow cytometry by staining positively selected cells with PE conjugated anti-mouse IgG2a (Southern Biotech, Birmingham, AL, USA), analyzing in a Gallios flow cytometer (Beckman Coulter), and found to be in the range of 95–98%. The CD14+ cells were subsequently grown in 6-well dishes at a density of 1.5×10^6 cells per well in RPMI medium supplemented with 10% FCS (Invitrogen, Carlsbad, USA). Cells were left over night at 37 °C in an atmosphere with 5% CO_2. The phenotypic morphology of cells, i.e. differentiation of monocytes into an early-stage adherent macrophage phenotype, was visualized and confirmed by phase contrast microscopy.

Bacterial infection

Two previously described *S. agalactiae* strains (ST103 and ST12) were obtained from The Norwegian Veterinary Institute [12]. These bovine adapted strains were originally isolated from milk samples. Bacteria were collected from

blood agar plates and grown in "Todd Hewitt broth" (Sigma-Aldrich) until mid-log phase. Growth was measured by optical density (OD) at 600 nm. The cultures were further aliquoted and frozen in 20% glycerol stocks in − 70 °C, and the final number of colony-forming units (CFU) was determined by serial dilutions and plating on blood agar plates. Bacteria used in this study all came from aliquots of the same batch.

For each individual animal the wells with immature macrophages were grouped into four classes with as equal number of wells and cells per class as possible. Two classes were infected with ST103 or ST12, in a multiplicity of infection (MOI) of 1 (1 bacterium per cell, on average). The third cell class (positive control) was exposed to 1 mg/mL of lipopolysaccharides (LPS, rough strains) from *Salmonella minnesota* Re 595 (Re mutant, Sigma-Aldrich) and the last class was left uninfected (negative control). After 1 h of exposure, 1% of penicillin/streptomycin (60 pg/mL penicillin and 100 μg/mL streptomycin) were added to prevent growth of remaining extracellular bacteria. The controls and the infected cells were treated equally. Inhibition of bacterial growth by antibiotics was verified by microscopy. Incubation was continued for one additional hour for LPS-exposed cells and for 5 more hours for bacteria infected and negative control cell classes, a total of 6 h incubation (Additional file 2: Figure S1). Media was aspirated and the cells were collected using cell scraper. Cells were centrifuged (400×g, 5 min), the pellet was washed with cold PBS buffer, snap frozen in liquid nitrogen and stored at − 70 °C.

RNA extraction and reverse transcription-quantitative PCR

Twenty-four RNA samples from six animals were used (Additional file 1: Table S1, Additional file 2: Figure S1). Total RNA was isolated from control and infected cells using the MirVANA isolation kit (Ambion, Austin, TX) following the manufacturer's instructions. All RNA samples were treated with amplification grade DNase I (Invitrogen) to remove any traces of genomic DNA. RNA concentration and quality was measured using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, USA) and 2100 BioAnalyzer (Agilent RNA 6000 Nano, Agilent Technologies, Palo Alto, USA), respectively. The RNA integrity numbers (RIN), concentrations and OD A260/A280 ratios are listed in Additional file 1: Table S1. For RT-qPCR a total of 200 ng RNA was used for cDNA synthesis using Tetro cDNA synthesis kit (Nordic BioSite, Norway), and cDNA equivalent to 5 ng of total RNA was used in qPCR reactions set up in triplicate for each sample using Express SYBR GreenER SuperMix with premixed ROX (Invitrogen) according to the manufacturer's recommendations. Transcript levels were analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the standard program: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by melting curve analyses. Gene-specific primers were either derived from literature or designed using Primer3 ver. 0.4.0 [15]. The transcript levels of the following genes were analyzed: *TNF-α*, *IL-1β*, *IL-6*, *IL-8*, *IL-10* and *TGFβ1*. The primer sequences are presented in Additional file 3: Table S2. The efficiencies of all primer pairs were tested by template dilution series using pooled cDNA from control and infected cells and were 100% (±10). Negative controls with no added template were included for all primer pairs (no template control), and no RT control reactions for each sample and each primer pair were run in qPCR in order to check for genomic DNA contamination (no RT control). *The peptidylprolyl isomerase A* (*PPIA*) housekeeping gene were used in the current study, as it has been shown to be one of the most stable genes for gene expression studies in cattle macrophages [14] and lymphocytes [16], and in human LPS-stimulated monocytes [17]. In the experiment, *PPIA* was expressed at the same level in the cells stimulated with both bacterial strains and LPS as in the negative controls. Initial analysis of the RT-qPCR data was performed using RQ Manager 1.2 (Applied Biosystems). Standard deviation of ≤0.3 per triplicate was accepted. The ΔCt method was used to calculate RT-qPCR data, i.e. $\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$, and normalized gene expression was calculated as $2^{(-\Delta Ct)}$. Fold change was calculated relative to the negative, unexposed control. Reciprocal values of fold change were used for down-regulated gene expression (i.e. *TGFβ1*) in order to facilitate the interpretation of the results. The differences of normalized gene expression levels between control and infected cells for each gene were tested using Wilcoxon matched-pairs signed rank test using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The pairwise differences between responses to LPS, ST103 and ST12 were tested using log-transformed fold change values for each treatment and gene by RM one-way ANOVA and Tukey's multiple comparison test using GraphPad Prism. The significance level was determined at $P < 0.05$.

MicroRNA sequencing and data analyses

Twenty RNA samples from six animals were used (control = 6, LPS = 4, ST12 = 5, ST103 = 5; see Additional file 1: Table S1 and Additional file 2: Figure S1 for details) for short non-coding RNA deep sequencing. Total RNA was isolated from control and infected cells using the MirVANA isolation kit, which retains short RNA fragments, and RNA concentration and quality were measured as described above for RT-qPCR (see Additional file 1: Table S1 for details). RNA-seq libraries were prepared with NEBNext® Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs), according

to the manufacturer's protocol. Libraries were sequenced (75 bp single-end, 3–400 million reads) on one lane with an Illumina NextSeq 500 machine. Preparation of RNA-seq libraries and sequencing were performed by the Norwegian Sequencing Centre (Oslo, Norway; http://www.sequencing.uio.no/).

The miRNA sequence data was analyzed using Oasis ver. 2.0, a web application that allows for fast and flexible online analysis of small-RNA-seq (sRNA-seq) data ([18]; http://oasis.dzne.de). Preliminary quality control analysis of the 20 fastq files was carried out with FASTQC software ver. 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Cutadapt ver. 1.2.1 (https://cutadapt.readthedocs.io/en/stable/) was then used to trim 3′ adaptor sequences. Reads, shorter than 17 and longer than 32 nucleotides after trimming were discarded. Preprocessed reads, which successfully passed filtering were aligned to the bovine reference genome assembly (bostau8) using STAR ver. 2.30.0e_r291 [19] in non splice-junction-aware mode, allowing 5% mismatch of the read length (0 mismatches for reads with length 17–19, 1 mismatch for reads with length 20–32). Reads that could only be aligned using soft trimming (trimming of the beginning or end of reads that is not counted as mismatches) were removed from STAR's output using a custom script. Quantification of uniquely mapped reads was performed using featureCounts ver. 1.4.6 [20], and assignment to the short RNA species was performed using miRBase ver. 21 (release date: 2014–6-22) and Ensembl (v84).

In addition to profiling the expression of known miRNAs, the prediction of potentially novel miRNAs was performed by miRDeep2 ver. 2.0.0.5 software [21] on the combined set of all input fastq files (i.e., all the input samples were merged into a unique file containing the total reads). miRDeep2 analysis were performed with the default parameters except for the minimum read depth (–a). This parameter is automatically computed according to the total number of uniquely mapped reads, targeting a performance of at least 80% true positives and 80% recall. Further filters were applied in order to identify those novel miRNAs that have the highest likelihood of being true positives. These are based on significant randfold p-value (default mirdeep2 threshold), miRDeep2 score > 5, where both the mature and star read counts were expressed with a minimum of 5 reads each and rFAM alerts for other types of RNA (e.g. rRNAs and tRNAs). As Oasis maps to the mature miRNA sequence, miRDeep2 code was modified in order to show the mature miRNA position instead of the pre-mature miRNA position.

Differential expression analysis

In order to identify statistically significant differentially expressed (DE) miRNAs between control and treatment samples, DESeq2 ver. 1.4.5. [22] was applied. The analyses were performed pairwise, i.e. negative control and one of the treatment groups at the time (LPS, ST103 or ST12, respectively) were analyzed. In addition, a pairwise comparison between the two bacterial treatment groups (i.e. ST103 and ST12) was performed. The miRNA read counts identified by miRDeep2 were normalized across all samples using DESeq2 normalization method [22]. DE miRNAs were defined as having a Benjamini and Hochberg corrected P-value of < 0.05 and with the minimum number of miRNAs (mean expression values) with an average of 5 reads for either biological condition. Principal component analysis (PCA) and hierarchical clustering of the samples were performed in DESeq2, in order to determine how well samples cluster together based on the similarity of their sRNA expression. The analyses were performed using Oasis.

Target prediction and pathway analysis

DE miRNAs identified in ST103 ($n = 17$) vs. negative control and in ST12 ($n = 44$) vs. negative control were used as input lists in Ingenuity Pathway Analysis (IPA; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) in order to identify biological functions and networks that were overrepresented in the datasets.

Target genes that are potentially regulated by DE miRNAs were predicted using TargetScan ver.7.1 [23]. Given the high false positive rates for miRNA target prediction, we identified only those potential target genes that had cumulative weighted context++ score < – 0.5 [23].

Identification of statistically overrepresented biological pathways in the lists of target genes of the DE miRNAs were performed by pathway analysis using InnateDB, a curated database of innate immunity genes, pathways and molecular interactions (www.innatedb.com; [24]). Target genes predicted by TargetScan were converted to human homologs and further analyzed in InnateDB. The pathway overrepresentation analyses were performed using hypergeometric test and Benjamini and Hochberg corrected P-value ≤0.1 was defined as significant.

Results

Reverse transcription-quantitative PCR

To assess the efficiency and quality of inflammatory responses in vitro, we measured the transcription of six major macrophage-associated cytokines by RT-qPCR, following co-incubation of macrophages with either of the two *S. agalactiae* strains ST103 or ST12, LPS or control medium. *TNF-α*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* were significantly up-regulated by LPS, ST12 and ST103 compared to the controls ($P \leq 0.05$, Fig. 1). *TGFβ1* were significantly down-regulated only in the ST12 infected macrophages ($P \leq 0.05$, Fig. 1). *IL-6* and *IL-10* displayed significantly different responses both for LPS vs. ST103,

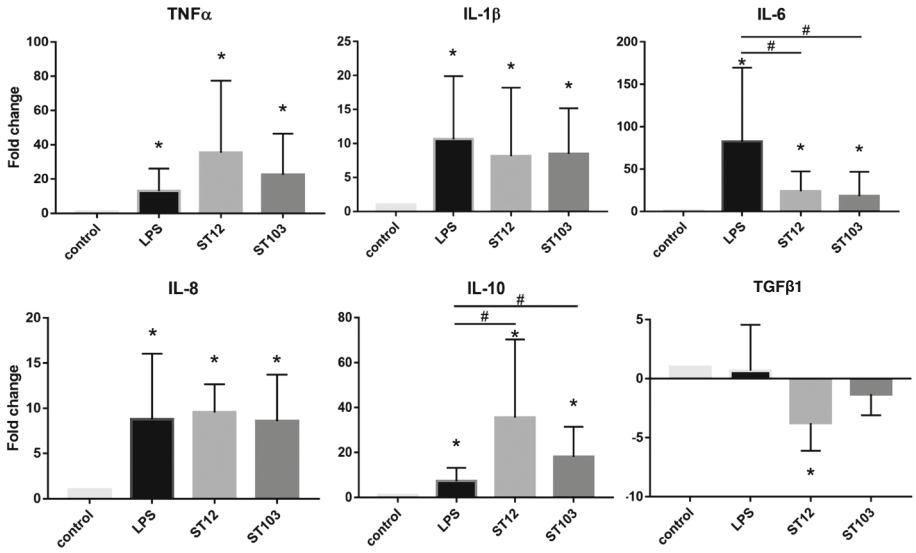

Fig. 1 mRNA gene expression levels in bovine monocyte-derived macrophages as determined by reverse transcription-quantitative PCR (RT-qPCR). Genes displaying a significant difference in expression between the *Streptococcus agalactiae* strains ST103 and ST12, and the lipopolysaccharides (LPS) stimulated cells, respectively, compared to uninfected controls are denoted with * ($P \leq 0.05$). The significant differences between responses to LPS vs. ST103, and LPS vs. ST12, respectively, are denoted with # ($P \leq 0.05$). Data are presented as fold change relative to negative control, and as a mean values with SD. *Tumor necrosis factor alpha* (*TNFα*), *interleukin-1 beta* (*IL-1β*), *interleukin-6* (*IL-6*), *interleukin-8* (*IL-8*), *interleukin-10* (*IL-10*) and *transforming growth factor beta 1* (*TGFβ1*). *Peptidylprolyl isomerase A* (*PPIA*) was used as a reference gene

and for LPS vs. ST12 ($P \leq 0.05$, Fig. 1). When comparing the two *S. agalactiae* strains we did not observe significantly different expression of any of the six cytokines.

Identification of small RNAs in bovine monocytes

Next, we conducted miRNA-seq on 20 sample libraries from the bovine monocyte-derived macrophages. Preliminary quality control analysis of the resulting 20 fastq files revealed that all libraries passed the quality criteria with a Phred score > 32. The sequencing resulted in a total of 356,741,900 high quality reads. Among them, 349,785,433 sequences (98%) remained after adaptor trimming. Approximately 43% of all reads were discarded due to length filtering after adaptor trimming. The proportion of reads uniquely aligning to different RNA biotypes demonstrated that miRNAs were the dominant class of small RNAs sequenced in our small RNA libraries. The majority (> 86%) of all reads in the 17 to 32 nucleotide fraction aligned to known miRNAs. Almost all the remaining reads mapped to small nucleolar RNAs (snoRNAs). The number of initial reads per sample, the percentage of trimmed reads and the percentage of uniquely mapped reads for each sample are presented in Additional file 4: Table S3.

The number of miRNAs with a normalized minimum mean of 5 reads detected in unchallenged macrophages were 249, while 243, 245 and 253 miRNAs were identified in LPS, ST12 and ST103 challenged macrophages, respectively (Additional file 5: Table S4). The highest expressed miRNA was bta-miR-21-5p, while four of the most abundant miRNAs belong to the bta-let-7 family (i.e., bta-let-7f, bta-let-7a-5p, bta-let-7 g and bta-let-7i). The top 10 most abundant miRNAs are presented in Fig. 2.

Applying the miRDeep2 algorithm to all 20 samples, we identified 31 high confidence, putatively novel bovine miRNAs (Table 1). Homology search in the miRBase database using the BLASTN method identified that 27 of the 31 novel miRNAs had 100% identity to known miRNAs in other species, one had a significant homology with the bta-miR-2285 family whereas three did not show significant homology to any other known miRNAs (Table 1). The high read counts observed for several of these predicted novel miRNAs suggests that the majority of them represent true novel bovine miRNAs.

Differentially expressed miRNAs in macrophages challenged with *S. agalactiae*

The difference between the negative control and the ST103 and ST12 infected samples, respectively, was confirmed by PCA of the small RNA sequence reads. For ST103 infected samples, PC1 explained 31% and PC2 explained 22% of the overall miRNA expression variability. While for ST12 infected samples, PC1 explained 39% and PC2 explained 20% of the overall miRNA expression variability. No clear difference between the negative control and the LPS challenged samples was revealed by PCA (Additional file 6: Fig. S2).

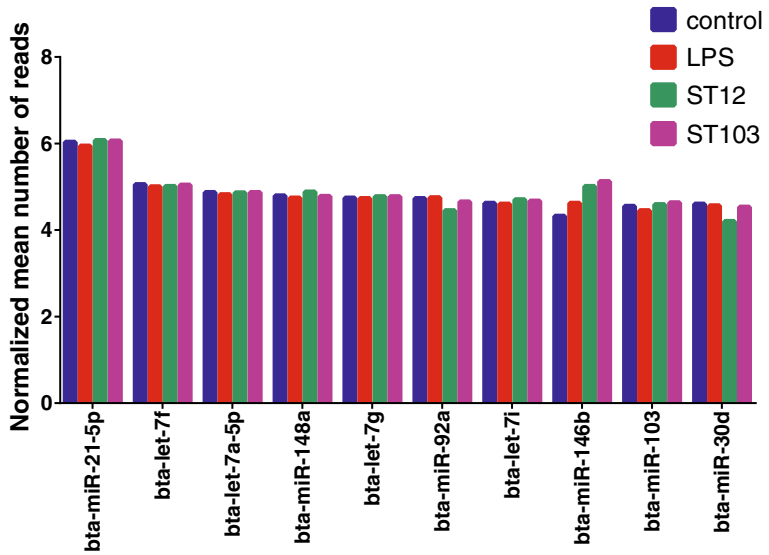

Fig. 2 The top ten most abundant miRNAs across all samples. Bovine monocyte-derived macrophages infected in vitro with *Streptococcus agalactiae* strains ST103 or ST12 or lipopolysaccharides (LPS), and uninfected negative controls. Mean miRNAs expression values (log transformed normalized mean number of reads) are presented on the x-axis, as calculated by DESeq2 software (at least 5 mean reads for either biological condition)

The analyses of differential expression revealed 17 DE miRNAs in macrophages challenged with ST103 and 44 DE miRNAs in macrophages challenged with ST12, compared to the respective unchallenged negative controls ($P < 0.05$) (Table 2 and more detailed information in Additional file 7: Table S5). Moreover, 13 DE miRNAs were detected when macrophages challenged with ST12 were compared to macrophages challenged with ST103 ($P < 0.05$) (Additional file 7: Table S5). No significantly differently regulated miRNAs were identified in LPS challenged macrophages compared to the unchallenged controls. Heatmap analysis of the top most significant DE miRNAs in the ST103 and the ST12 challenged samples, respectively, shows that the infected samples and most of the control samples cluster into different groups (Fig. 3).

Eleven DE miRNAs were commonly regulated by both *S. agalactiae* strains (6 up-regulated and 5 down-regulated), while 33 DE miRNAs were only regulated by ST12 (9 up-regulated and 24 down-regulated) and 6 were only regulated by ST103 (4 up-regulated and 2 down-regulated; Fig. 4). Ten out of 13 DE miRNAs between ST12 and ST103 were also found either among DE miRNAs in macrophages challenged with ST12 or with ST103, compared to the respective unchallenged negative controls. Moreover, three out of these (i.e. bta-miR-2478, bta-miR-1249 and bta-miR-2898; Additional file 7: Table S5) were found among DE miRNAs commonly regulated by both *S. agalactiae* strains.

One novel miRNA (p-bta-miR-3) was found to be DE in response to both bacterial strains (Fig. 4, Additional file 7: Table S5). This 20 nt long miRNA was mapped to bovine chromosome 9 and had a significant homology to mmu-mir-7025-5p (E-value 6.2).

Enriched pathways of differentially regulated miRNAs and their predicted target genes

IPA was used to analyze the miRNAs that were differentially regulated in response to ST103 infection ($n = 17$) in order to identify biological networks and functions enriched in the dataset. Nine bovine miRNAs were mapped to their human miRNA homologs. The analyses revealed that the most significant biological network in the dataset with 6 focus molecules, were associated with cancer, organismal injury and abnormalities and reproductive system disease (Fig. 5a, Additional file 8: Table S6). MiRNAs differentially regulated in response to ST12 infection ($n = 44$) were also analyzed. Twenty-nine bovine miRNAs were mapped to their human miRNA homologs by IPA. The analyses revealed that the most significant biological network with 13 focus molecules were associated with connective tissue disorders, inflammatory disease and inflammatory response (Fig. 5b, Additional file 8: Table S6). Due to difficulties in accurately predicting miRNA targets, it is more common to examine the statistically overrepresented functional categories among predicted target genes than focusing on individual gene predictions. In order to identify significantly enriched pathways among the target genes of the DE miRNAs, we analyzed the up- and down-regulated target genes separately. Hence, 239 and 182 target genes of the down- and up-regulated DE miRNAs, respectively, in response to ST103, and 1003 and 463 target genes of down- and up-regulated DE miRNAs, respectively, in response to ST12 were analyzed using InnateDB. The top significantly

Table 1 Novel microRNAs identified in bovine monocyte-derived macrophages with or without challenge with lipopolysaccharides (LPS), *Streptococcus agalactiae* strains ST103 and ST12

provisional ID	mature read count	star read count	miRDeep2 score	example miRBase miRNA with the same seed	consensus mature sequence	precursor coordinate
chr25_2078	63,905	8,00	32,589,40	mmu-miR-106b-3p	ccgcacuguggguacuugcugc	chr25:36892057..36892117:+
chr8_3368	48,256,00	44,00	24,641,00	hsa-let-7d-3p	cuauacgaccugcugccuuucu	chr8:86887438..86887514:+
chrX_3864	40,373,00	6,00	20,590,60	hsa-miR-223-5p	cguguauuugacaagcugaguug	chrX:99936328..99936387:-
chr7_3248	14,956,00	13,00	7636,00	mmu-miR-24-1-5p	gugccuacugagcugaaacacag	chr7:12981645..12981702:-
chr26_2162	10,315,00	54,00	5290,60	eca-miR-146b-3p	ugcccuagggacucaguucuggu	chr26:22930915..22930975:+
chr11_440	4397,00	11,00	2251,80	ssc-miR-181d-3p	accaccgaccguugacuguacc	chr11:95709449..95709509:+
chr8_3359	3633,00	95,00	1990,00	mmu-miR-27b-5p	agagcuuagcugauuggugaaca	chr8:83009841..83009902:+
chr25_2117	3826,00	7,00	1958,50	gra-miR7486h	cagcaacuaaagaucccucagg	chr25:34409297..34409357:-
chr22_1816	3325,00	78,00	1741,70	bmo-miR-3000	cugcgcuuggauuucguuccc	chr22:51543484..51543548:+
chr3_2544	1854,00	9,00	951,00	bta-miR-2285f	aaaaaccugaaugacccuuuug	chr3:94548590..94548649:+
chr8_3366	1064,00	754,00	932,60	hsa-let-7a-3p	cuauacaaucuauugccuuccc	chr8:86885231..86885309:+
chr7_3219	1392,00	74,00	748,70	hsa-miR-378a-5p	cuccugacuccagguccugugu	chr7:63067305..63067362:+
chr3_2643	863,00	137,00	513,90	hsa-miR-30c-2-3p	cugggagaggguuguuuacucc	chr3:106059376..106059437:-
chr16_1041	937,00	11,00	486,50	hsa-miR-181a-3p	accaucgaccguugauuguacc	chr16:79685955..79686018:-
chr14_712	672,00	34,00	364,10	hsa-miR-30a-3p	cuuucagucagauguuugcugcu	chr14:8080297..8080360:+
chr1_143	643,00	10,00	335,60	mmu-miR-15b-3p	cgaaucauuauuugcugcucuag	chr1:107923396..107923457:-
chr12_549	565,00	17,00	301,20	hsa-miR-92a-1-5p	agguugggaucgguugcaaugcu	chr12:66227265..66227321:+
chr1_111	475,00	6,00	249,50	mmu-miR-125b-2-3p	acaagucaggcucuugggacc	chr1:19881359..19881419:-
chr16_1003	359,00	103,00	240,00	efu-miR-9283	uguggccucuggguguguacccuc	chr16:33022297..33022356:-
chrX_3750	463,00	6,00	240,00	ssc-miR-374a-3p	uuaucagguuguauuguaauu	chrX:81951234..81951286:+
chr18_1154	297,00	42,00	181,00	hsa-let-7e-3p	cuauacggccuccuagcuuucc	chr18:58015043..58015110:+
chr4_2734	287,00	41,00	168,50	–	acacgcguccuuggauccugacu	chr4:119142851..119142912:+
chrX_3767	228,00	37,00	139,50	hsa-miR-222-5p	cucaguagccaguguagaucc	chrX:103538171..103538234:+
chrX_3861	197,00	16,00	113,20	hsa-let-7a-3p	cuauacaacuuacuacuuuccc	chrX:96382645..96382725:-
chr19_1372	157,00	5,00	82,60	–	agggagucccugguaguucagu	chr19:46741763..46741851:-
chr19_1308	103,00	22,00	65,00	–	ccccggcuuuuccucccccagg	chr19:62308493..62308542:+
chr5_2875	86,00	8,00	52,80	ssc-miR-7134-5p	auguccgcggguucccugucc	chr5:112083860..112083922:+
chr19_1281	70,00	5,00	43,00	hsa-miR-152-5p	agguucugugauacacuccgacu	chr19:39081179..39081236:+
chr18_1135	13,528,00	95,00	5,60	mmu-miR-140-5p	cagugguuuuacccuaugguag	chr18:37088153..37088219:+
chr19_1237	93,00	15,00	5,40	ahy-miR3511-5p	accagggcuggaagcugcuucu	chr19:9534051..9534107:+
chr2_1498	1928,00	20,00	5,30	hsa-miR-26b-3p	ccuguucuccauuacuuggcucg	chr2:107133408..107133466:+

enriched pathways are presented in Table 3. Target genes and pathways for each list of DE miRNAs are presented in Additional file 9: Table S7.

Discussion

MiRNAs are suggested to be fine-tuners of gene expression during inflammatory response [25, 26]. This indicates a great potential of the genome-wide miRNome profiling for investigation of inflammation. In this study, we have identified the early phase miRNome of bovine monocyte-derived macrophages infected in vitro with two strains of *S. agalactiae* derived from Norwegian dairy herds. *S. agalactiae* is a major causative agent of subclinical mastitis and an increasing problem in Norway. Furthermore, *S. agalactiae* is widely recognized as a main cause of life-threatening infections in human neonates, pregnant females, and elderly adults [27]. The two strains used here differ in their ability to survive in the environment and transmit within dairy herds. ST103 is the most predominant isolate in bovine mastitis found in farms with substantial environmental contamination, while ST12 was found in cattle herds with no positive environmental samples [12]. Using modern sequencing of miRNAs from macrophages infected with *S. agalactiae* and analyzing over 356

Table 2 MicroRNAs significantly differentially expressed between bovine monocyte-derived macrophages infected with *Streptococcus agalactiae* strains ST103 or ST12 and the respective uninfected controls

ST103			ST12		
miRNA ID	Log2FC	*P*-value	miRNA ID	Log2FC	*P*-value
p-bta-miR-3	8,22	8,77E-45	p-bta-miR-3	9,98	1,10E-53
bta-miR-2284i	7,37	2,28E-29	bta-miR-2284i	8,62	9,53E-41
bta-miR-2285m	4,97	5,87E-13	bta-miR-2438	7,93	2,46E-25
bta-miR-7858	4,24	1,15E-06	bta-miR-2285 m	6,08	9,41E-17
bta-miR-222	1,75	1,05E-05	bta-miR-2478	−2,29	4,69E-15
bta-miR-146b	2,34	1,88E-04	bta-miR-223	1,27	3,63E-14
bta-miR-2427	−2,53	1,60E-03	bta-miR-1249	−3,29	1,77E-13
bta-miR-2898	−1,52	2,88E-03	bta-miR-128	−1,91	2,34E-11
bta-miR-2478	−0,93	4,00E-03	bta-miR-2427	−4,19	1,55E-09
bta-miR-628	1,20	6,59E-03	bta-miR-2898	−2,92	2,98E-09
bta-miR−1306	−1,73	8,43E-03	bta-miR-500	1,39	4,76E-09
bta-miR-1249	−1,54	1,14E-02	bta-miR-92b	−2,27	2,08E-08
bta-miR-708	1,08	1,85E-02	bta-miR-484	−1,81	3,88E-08
bta-miR-221	0,69	3,71E-02	bta-miR-365-3p	−2,36	4,13E-07
bta-miR-1246	−0,94	4,33E-02	bta-miR-1306	−3,34	8,34E-07
bta-miR-2892	− 1,57	4,97E-02	bta-miR-374b	1,57	6,66E-06
bta-miR-9-5p	1,13	4,97E-02	bta-miR-628	1,50	7,96E-06
			bta-miR-197	−2,21	9,36E-06
			bta-miR-425-5p	0,93	3,38E-05
			bta-miR-340	−1,39	1,28E-04
			bta-miR-30d	−1,31	1,72E-04
			bta-miR-425-3p	1,04	1,82E-04
			bta-miR-505	−1,15	1,19E-03
			bta-miR-125a	−2,01	1,22E-03
			bta-miR-146b	2,11	1,64E-03
			bta-miR-1343-3p	−1,59	1,64E-03
			bta-miR-2388-5p	−2,67	2,77E-03
			bta-miR-423-3p	−0,98	2,85E-03
			bta-miR-328	−1,84	3,39E- 03
			bta-miR-30b-5p	1,26	3,63E-03
			bta-miR-92a	−0,91	6,29E-03
			bta-miR-1468	−0,88	6,32E-03
			bta-miR-30f	−1,34	6,58E-03
			bta-miR-125b	−1,18	8,86E-03
			bta-miR-10a	−0,76	9,31E-03
			bta-miR-2431-3p	−2,25	9,76E-03
			bta-miR-155	1,33	1,76E- 02
			bta-miR-361	−0,97	1,97E-02
			bta-miR-122	2,12	2,12E-02
			bta-miR-221	0,65	2,16E-02
			bta-miR-2284ab	−1,06	3,02E-02

Table 2 MicroRNAs significantly differentially expressed between bovine monocyte-derived macrophages infected with *Streptococcus agalactiae* strains ST103 or ST12 and the respective uninfected controls *(Continued)*

ST103			ST12		
miRNA ID	Log2FC	*P*-value	miRNA ID	Log2FC	*P*-value
			bta-miR-2284w	−0,74	3,02E-02
			bta-miR-30c	−0,82	3,62E-02
			bta-miR-669	−0,85	4,39E-02

Log2FC – log2 fold change values compared to controls, *P*-value - Benjamini and Hochberg corrected *P*-value

million reads, we found that several miRNAs were significantly differentially regulated in response to infection (Figs. 3 and 4). We observed that fewer miRNAs were differentially regulated in macrophages challenged with strain ST103 than with strain ST12 (17 and 44, respectively; Fig. 4). This suggests that ST103 induces relatively subtle changes in miRNA expression during the early stage of infection. However, the expression pattern of the examined pro- and anti-inflammatory genes was not significantly different between these two strains, except for *TGFβ1*, that was significantly down-regulated only by ST12 (Fig. 1).

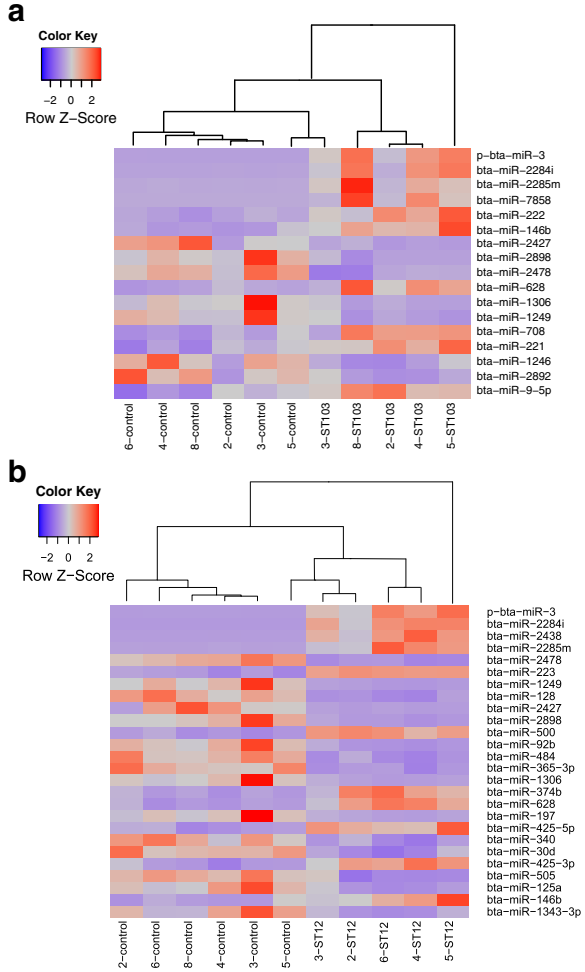

Fig. 3 Heatmap of the top most significant differentially expressed microRNAs ($P < 0.05$) between bovine monocyte-derived macrophages infected in vitro by **a**) *Streptococcus agalactiae* strain ST103 or **b**) *Streptococcus agalactiae* strain ST12 and non-infected negative controls, as determined by DESeq2 analyses. Red color indicates high expression of the microRNA

It has been shown that different bacterial strains are often associated with a variety of virulence factors, which, in turn, are associated with the outcome of infection in the host [28, 29]. The latest study of molecular and virulence characteristics of the most prevalent bovine *S. agalactiae* isolates has shown that these factors in strain ST103 (but also in other of the most prevalent bovine isolates) are associated with growth ability in milk, biofilm formation and adhesion to bovine mammary epithelial cells. Furthermore, ST103 showed significantly higher hemolytic activity and cytotoxicity compared to most of the other bovine strains [30]. On the contrary, the characterization of ST12 revealed that this strain shows high rates of resistance to erythromycin and clindamycin [31] and can survive within macrophages [32]. The observed differences in the miRNA expression profiles during the early stage of macrophage infection may be explained by the different characteristics of these two *S. agalactiae* strains such as the differences in virulence factors, the different niches and modes of transmission.

Both strains increased the level of miR-146b and IL-10 transcripts. This may be associated with toll-like receptor (TLR) recognition of bacterial patterns, as shown in monocytes for other bacterial TLR-agonists. MiR-146b has been reported to reduce the expression of several pro-inflammatory cytokines and chemokines in human monocytes upon stimulation with LPS [33], which suggests that miR-146b has an anti-inflammatory activity in monocytes. In a previous transcriptomic study, we found indications that another gram-positive bacterial mastitis pathogen, *S. aureus*, promotes bovine macrophages to be activated in an alternative manner [14]. Here we observed several indications of alternative activation on the miRNA level as a result of streptococcal uptake in macrophages. Both strains induced miR-221, ST12 induced miR-30b, miR-223, miR-374b and miR-500 but down-regulated miR-125a and miR-125b, while ST103 induced miR-222, all associated with alternative macrophage activation [34–37].

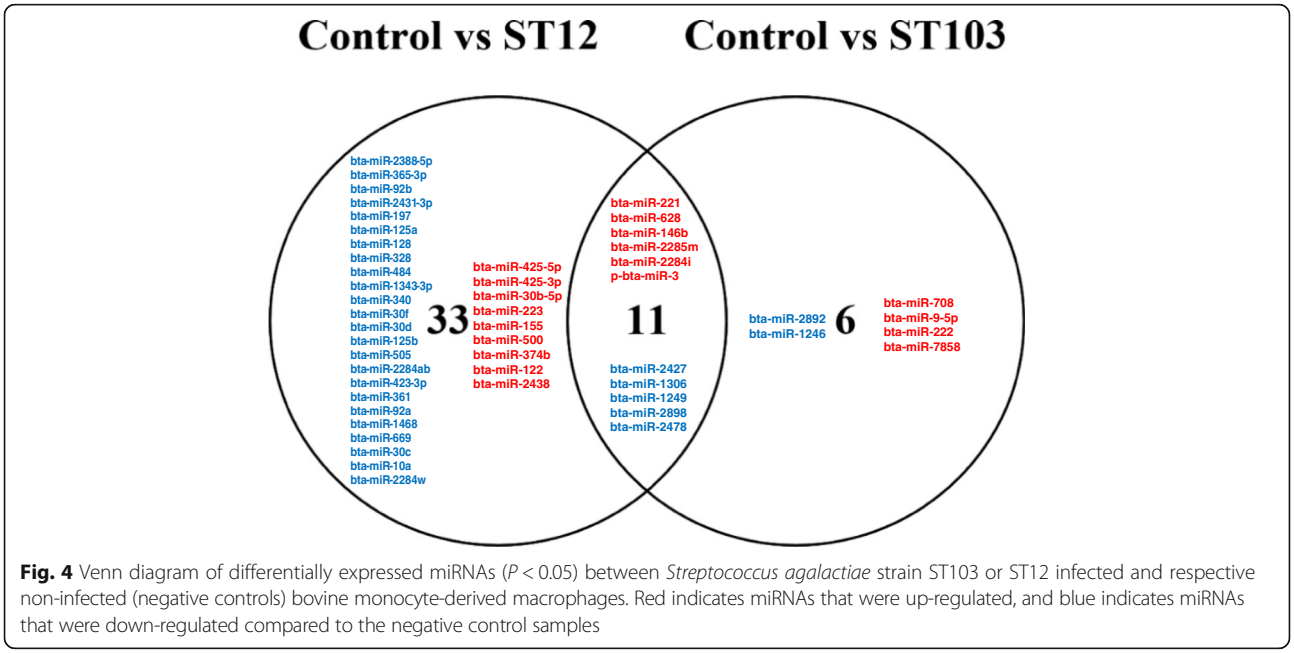

Fig. 4 Venn diagram of differentially expressed miRNAs ($P < 0.05$) between *Streptococcus agalactiae* strain ST103 or ST12 infected and respective non-infected (negative controls) bovine monocyte-derived macrophages. Red indicates miRNAs that were up-regulated, and blue indicates miRNAs that were down-regulated compared to the negative control samples

While one study reported that miR-125a promotes classical macrophage activation [38], and several of the DE miRNA found in our study are not accounted for in the literature, the results suggest that *S. agalactiae* may induce an alternative-like macrophage activation similar to *S. aureus*. However, this result needs to be confirmed by more comprehensive functional studies.

One of the novel miRNA (p-bta-miR-3) was found to be DE in response to both bacterial strains (Fig. 4). This miRNA shares 100% sequence identity and covers 95% of the query (i.e. 19 out of 20 nucleotides match) when blasted against the genome sequences of several *S. agalactiae* strains (e.g. GenBank: CP025028.1; https://blast.ncbi.nlm.nih.gov/Blast.cgi). This suggests that it may be

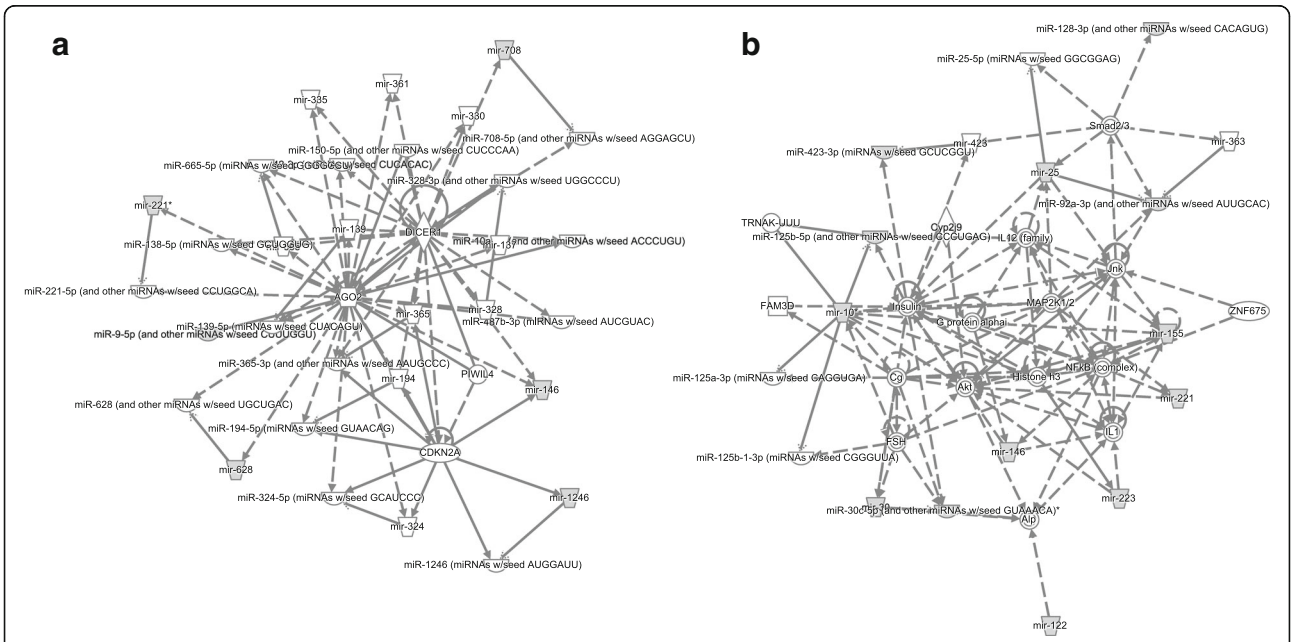

Fig. 5 Functional network overrepresented in the list of differentially expressed miRNAs in response to infection of bovine macrophages with *Streptococcus agalactiae* strain (**a**) ST103 and (**b**) ST12. Ingenuity Pathway Analysis (IPA) identified 6 associated molecules with a network score of 16 for ST103 and 13 associated molecules with a network score of 31 for ST12

Table 3 Top significant pathways overrepresented among target genes of differentially expressed miRNAs in response to exposure of bovine macrophages in vitro to *Streptococcus agalactiae* strains ST103 or ST12

		Pathway name	*P*-value	*P*-value (B-H)	Genes
ST103	miRNAs up-regulated	Mucin type O-Glycan biosynthesis	1.4E-4	0.01	GALNT3; GALNT4; GALNT9; POC1B-GALNT4;
		GABA receptor activation	1.0E-4	0.02	GABRA1; GABRB2; GNG10; GNG5; KCNJ2;
		Regulation of gene expression in early pancreatic precursor cells	0.001	0.06	ONECUT1; ONECUT3;
	miRNAs down-regulated	Integrin signaling pathway	0.004	0.10	ACTN1; ACTN2; BCR; CAV1; FYN; ITGA1; MAPK8; PXN; SOS1; TNS1; VCL;
		Sodium/Calcium exchangers	0.003	0.11	SLC24A1; SLC24A2; SLC24A3; SLC24A4; SLC8A1; SLC8A2; SLC8A3;
		Reduction of cytosolic Ca++ levels	0.004	0.11	ATP2A2; ATP2B1; ATP2B2; ATP2B3; ATP2B4; SLC8A1; SLC8A2; SLC8A3;
ST12	miRNAs up-regulated	Activation of G protein gated Potassium channels	2.3E-4	0.04	GNB3; GNG10; GNG2; GNG5; KCNJ6;
		G protein gated Potassium channels	2.3E-4	0.04	GNB3; GNG10; GNG2; GNG5; KCNJ6;
		Inhibition of voltage gated Ca2+ channels via Gbeta/gamma subunits	2.3E-4	0.04	GNB3; GNG10; GNG2; GNG5; KCNJ6;
	miRNAs down-regulated	IL4-mediated signaling events	5.1E-4	0.15	BCL2L1; CCL11; CCL26; FCER2; IL10; IRF4; IRS2; SOCS1; SOCS5; STAT6;
		Intrinsic Pathway for Apoptosis	3.6E-4	0.16	BAK1; BAX; BCL2L1; BMF; CASP7; TFDP1; YWHAB; YWHAG;
		Role of parkin in ubiquitin-proteasomal pathway	3.1E-4	0.28	PARK2; UBE2E2; UBE2G1; UBE2L3;

GALNT3/4/9 - polypeptide N-acetylgalactosaminyltransferase 3/4/9; POC1B - POC1 centriolar protein B; GABRA1/B2 - gamma-aminobutyric acid type A receptor alpha1 subunit/beta2 subunit; GNG 2/5/10 - G protein subunit gamma 10/5; KCNJ2/6 - potassium voltage-gated channel subfamily J member 2/6; ONECUT1/3 - one cut homeobox 1/3; ACTN1/2: actinin alpha 1/2; BCR - RhoGEF and GTPase activating protein; CAV1 - caveolin 1; FYN - FYN proto-oncogene, Src family tyrosine kinase; ITGA1 - integrin subunit alpha 1; MAPK8 - mitogen-activated protein kinase 8; PXN - paxillin; SOS1 - SOS Ras/Rac guanine nucleotide exchange factor 1; TNS1 - tensin 1; VCL - vinculin; SLC24A1/2/3/4 - solute carrier family 24 member 1/2/3/4; SLC8A1/2/3 - solute carrier family 8 member 1/2/3; ATP2A2 - ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2; ATP2B1/2/3/4 -ATPase plasma membrane Ca2+ transporting 1/2/3/4; GNB3 - G protein subunit beta 3; BCL2L1- BCL2 like 1; CCL11/26 - chemokine (C-C motif) ligand 11/26; FCER2 - Fc fragment of IgE receptor II; IL10 - interleukin 10; IRF4 - interferon regulatory factor 4; IRD2 - insulin receptor substrate 2; SOCS1/5 - suppressor of cytokine signaling 1; STAT6 - signal transducer and activator of transcription 6; BAK1 - BCL-2 antagonist killer 1; BAX - BCL-2 associated X; BMF - Bcl2 modifying factor; CASP7 - caspase 7; TFDP1 - transcription factor Dp-1; YWHAB/G - tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta/gamma; PARK2 - parkin RBR E3 ubiquitin protein ligase; UBE2E2/G1/L3 - ubiquitin-conjugating enzyme E2E2/E2G1/E2L3

a bacterial-derived miRNA released by infection of the macrophages. Bacterial-derived miRNAs have recently been extensively studied [39, 40]. It is likely that these small RNA molecules may repress the ability of the host cell to resist bacterial invasion, as has been shown for viruses and fungi [41–43]. It is not clear if bacterial miRNAs require the host cell miRNA processing machinery for its biogenesis or if it is a bacterial RNA fragment released into the host cell cytoplasm during bacterial lysis. Whether p-bta-miR-3 observed in our study is an authentic miRNA of bacterial origin needs to be further tested.

Mapping of bovine miRNAs to their human homologs may reveal conserved pathways and biological functions associated with the regulation of gene expression during immune response to bacteria. To characterize the distinct DE miRNA profiles of the two bacterial strains we performed pathway analysis using IPA. As a result 2 genes, *dicer 1 ribonuclease III* (*DICER1*) and *argonaute 2* (*AGO2*), were found to be associated with the ST103 DE miRNAs (e.g., miR-9-5p and miR-708; Fig. 5a). *DICER* and *AGO2* are miRNA processing enzymes, and *DICER1* knockdown cell lines and animals have been correlated with a global drop of mature miRNA expression levels, shifting macrophages towards a classically activated phenotype [44–46]. These results show that strain ST103 induces miRNAs that likely contribute to maintain an alternative macrophage phenotype.

For miRNAs down-regulated in ST103 infected macrophages, putative target genes with a role in the integrin signaling pathway may be noted (Table 3). Integrin activation is necessary for leukocyte arrest before migration across the endothelial cell barrier and many bacterial species, such as group A streptococci [47] *S. aureus* [48], and *E. coli* [49] use host integrins for adhering to and invading host cells. Moreover, the study of virulence factors of bovine *S. agalactiae* isolates has identified virulence factors associated with adhesion ability to bovine mammary epithelial cells in strain ST103 [30]. These findings, together with our results may indicate that the integrin signaling pathway is essential for *S. agalactiae* ST103 strain invasion of macrophages or mammary epithelial cells.

Furthermore, target genes of ST103 up-regulated miRNAs are involved in gamma-aminobutyric acid (GABA) receptor activation (Table 3). GABA plays an inhibitory

role in autoimmune inflammation [50] and *GABA* receptor transcripts are present in different immune cells [50–53]. Inflammatory cytokine production in peripheral macrophages and T cell autoimmunity is decreased after GABA treatment [51, 52]. Moreover, GABA and GABA type A receptor agonists reduced cytotoxic immune responses of T cells [54]. Other target genes associated with up-regulated ST103 miRNAs belong to cancer, organismal injury, abnormalities, and reproductive system disease pathways, without obvious connection to the bacterial infection context (Additional file 8: Table S6). Taken together, ST103 induced miRNA regulation of integrin and GABA signaling, and maintenance of alternative macrophage activation suggest that miRNAs may be involved in the pathogenesis of subclinical mastitis. However, whether this is an evasion strategy developed by *S. agalactiae* ST103 to avoid host defense and promote its intracellular replication and persistence remains to be determined.

In contrast, IPA revealed that strain ST12 induced DE miRNAs were associated with inflammatory disease and inflammatory response (Fig. 5b, Additional file 8: Table S6). A number of genes associated with the ST12 induced miRNA networks are key players of immune response, i.e. *IL-1*, *nuclear factor kappa β* (*NFKβ*), *interleukin-12* (*IL-12*), *Janus kinase* (*JaK*) and *SMAD family member 2/3* (*Smad2/3*) (Fig. 5b). For three of the ST12-unique DE miRNAs (bta-miR-155, bta-miR-125b and bta-miR-223) evidence of induction by bacteria has been previously documented. It has been shown that miR-155 and miR-125b are involved in regulation of *TNF* production during mycobacterial infection [55, 56]. MiR-155 was reported to induce toll-like receptor 2 (TLR-2) recognition of *Streptococcus pneumoniae*, and promote effective bacterial clearance in the nasopharynx [57]. MiR-223 regulates inflammation (reviewed in [58]) and was found to be up-regulated in bovine mammary tissue infected with *S. uberis* [59]. Moreover, an earlier study shows that *Salmonella* can up-regulate intestinal epithelial miR-128 expression, which, in turn, decreases macrophage recruitment [60]. Finally, IPA revealed that miR-128 and miR-92a are associated with Smad2/3 proteins (Fig. 5b), critical downstream mediators of TGFβ-signaling (reviewed in [61]). This is consistent with the qPCR findings of down-regulation of *TGFβ1* by ST12 (Fig. 1).

The predicted target genes of the down-regulated miRNAs in ST12 infected macrophages were significantly enriched for genes with roles in interleukin-4 (IL-4)-mediated signaling events and intrinsic pathway for apoptosis (Table 3). These genes have multiple roles in regulation of apoptosis [e.g. *BCL-2 like 1* (*BCL2L1*), *BCL-2 antagonist killer 1* (*BAK1*), *BCL-2 associated X* (*BAX*)] and anti-inflammation (e.g. *IL-10*), and display chemotactic activity for eosinophils [e.g. *C-C motif chemokine ligand 11* (*CCL11*), *C-C motif chemokine ligand 26* (*CCL26*)]. Pro-apoptotic *BAK1* is a direct target of miR-125b [62] and in our study miR-125b was found to be down-regulated in response to ST12 (Fig. 4). Apoptosis plays a critical role in the pathogenesis of sepsis [63] and *S. agalactiae* strains are frequent agents of life-threatening sepsis and meningitis in human neonates and adults. This may indicate the putative mechanisms of pathogenesis, i.e. impaired immune responses due to extensive death of immune system cells [63]. However, further in vivo studies are needed in order to confirm this hypothesis.

The predicted target genes of ST12 up-regulated miRNAs have multiple roles in G protein signaling (Table 3). It has been shown that G-protein-coupled receptors (GPCRs) are linked to heterotrimeric G-proteins composed of α, β, and γ subunits (reviewed in [64]). Inflammatory cells such as macrophages express a large number of GPCRs for classic chemoattractants and chemokines, and these receptors are critical for both enhancement of inflammation and promotion of its resolution (reviewed in [65]). This may suggests that ST12-mediated up-regulation of miRNAs, which in turn regulate G-protein genes, may have impact on regulation of inflammatory gene expression, particularly those facilitating chemotaxis. Taken together, we found that strain ST12 induces a stronger inflammatory immune response in bovine macrophages than strain ST103, with miRNAs associated with down-regulation of the TGFβ/Smad signaling, chemotactic pathways and enhancement of apoptotic pathways and alternative macrophage activation.

Three out of thirteen DE miRNAs (i.e. bta-miR-2478, bta-miR-2898 and bta-miR-1249) detected among macrophages challenged with ST12 compared to macrophages challenged with ST103 were also found among DE miRNAs commonly regulated by both *S. agalactiae* strains (Fig. 4 and Additional file 7: Table S5). It was reported recently that bta-miR-2478 inhibits *TGFβ1* expression during mammary gland development in goats and a strong negative correlation between miR-2478 and *TGFβ1* expression was demonstrated [66]. We could not observe this negative correlation in our study, as the lowest transcript level of *TGFβ1* (Fig. 1) was associated with the lowest expression of bta-miR-2478 (Additional file 5: Table S4) in macrophages challenged with ST12, compared to the other groups. However, it has also been reported that this miRNA is associated with energy metabolism and feeding-induced changes in muscles, and regulates different target genes in cattle [67, 68]. This suggests that bta-miR-2478 may target other gene(s) than *TGFβ1* in infected macrophages. Bta-miR-2898 was found to be up-regulated in the mammary gland tissues of mastitis-infected cows, while bta-miR-1249 was identified as miRNA, which targets genome of H5N1 influenza A virus during in vitro infection [69, 70]. The significantly stronger down-regulation of these three miRNAs in macrophages challenged with ST12 compared to ST103 may be explained partly by the differences in virulence factors between these strains and suggest an

important role of these miRNAs during the early stage of macrophage infection.

We were not able to identify any significant DE miRNAs in response to LPS in our study, however, several pro- and anti-inflammatory genes were up-regulated after exposing macrophages to LPS (Fig. 1). This may be due to higher individual variation in miRNA expression between LPS-treated samples combined with lower number of biological replicates ($n = 4$) compared to ST12 and ST103 (Additional file 6: Figure S2). However, we were not able to identify any outliers within the LPS-treated group based on the variation pattern (Additional file 6: Figure S2), in order to improve the statistical analysis. The number of biological replicates needed to ensure valid biological interpretation of high-throughput sequencing results is unclear. However, some studies attempt to answer this question and provide guidelines for experimental design. Earlier it has been proposed that the absolute minimum of three biological replicates should be used for next-generation sequencing studies, however, Schurch et al. [71] suggested that at least six biological replicates should be used in order to assure statistical power in the detection of differentially expressed genes. In the present study, we used four replicates for LPS-stimulated and five replicates for each of the bacterial strain-challenged macrophages. Furthermore, using five biological replicates for ST103- and ST12-treated samples may also have a negative impact on the number of detected significant miRNAs. Increasing the number of biological replicates would be beneficial to increase the statistical power and accuracy of the RNA and miRNA-seq analyses, but unfortunately, this was not possible within the scope of the present project. Originally, we had planned for six samples of each class of exposure, but some of the cultures failed to yield sufficient number of cells.

We observed approximately 250 miRNAs expressed in bovine macrophages (Additional file 5: Table S4). This is in agreement with the previous reports on miRNA sequencing of primary bovine mammary epithelial cells infected with *S. uberis* [6] and with *S. aureus* and *E. coli* [7], but it is approximately 3 times more than identified in bovine alveolar macrophages [72]. In our study, some miRNAs were expressed at very high levels (Fig. 2), while the majority were expressed at low levels. This is also observed in several previous studies of macrophages and epithelial cells miRNome [6–8, 72].

Conclusions

Our study have shown that *S. agalactiae* strain ST12 induces miRNAs associated with a stronger inflammatory response than *S. agalactiae* strain ST103. Both seem to drive the macrophages away from a classical (M1) type of activation, but further studies are needed to classify them into canonical activation phenotypes. Taken together these analyses suggest that the differentially expressed miRNAs identified in this study during in vitro infection of bovine macrophages with two different strains of *S. agalactiae* likely are crucial regulators of the innate immune response to this pathogen, and thus represent potential biomarkers of infection and inflammation. We have also identified several candidate pathways likely involved in the pathogenesis of subclinical mastitis. The study contributes to better understanding of the pathogenic mechanisms of different bacteria strains within one species.

Additional files

Additional file 1: Table S1. RNA samples that were used in the study. RIN - RNA integrity number; RT-qPCR - reverse transcription-quantitative PCR; miRNAs seq - microRNA sequencing. X indicates which samples were used in each of the experiments. (DOCX 16 kb)

Additional file 2: Figure S1. Experimental design of reverse transcription-quantitative PCR (RT-qPCR) and microRNA sequencing experiments. S. agal: samples of in vitro exposure of blood monocyte-derived macrophages with live *Streptococcus agalactiae* strain ST103 or strain ST12, respectively; Neg.con: negative control: sample of uninfected blood monocyte-derived macrophages; 6 h – 6 h infection with *Streptococcus agalactiae*, 2 h – 2 h exposure to LPS. For details on the experimental design, see the Materials and Methods section. (PDF 134 kb)

Additional file 3: Table S2. List of primers used for reverse transcription-quantitative PCR (RT-qPCR). (DOCX 14 kb)

Additional file 4: Table S3. The results of next generation sequencing of 20 libraries from bovine monocyte-derived macrophages. The number of initial reads per sample, the percentage of trimmed reads, the percentage of uniquely mapped reads, read distribution (% reads), length filtering (% of discarded reads) and an average read length (nt) for each sample are presented. (XLSX 14 kb)

Additional File 5: Table S4. The lists of microRNAs identified in blood monocyte-derived macrophages infected in vitro with live *Streptococcus agalactiae* strains ST103 and ST12, LPS, and uninfected (controls), respectively. Mean reads number normalized across all samples were calculated using DESeq2. (XLSX 71 kb)

Additional file 6: Figure S2. Principal component analysis of mapped sequence reads. Control represents sequences from non-infected bovine monocyte-derived macrophage libraries, and a) *Streptococcus agalactiae* strain ST103 infected; b) *Streptococcus agalactiae* strain ST12 infected; c) LPS-challenged; and d) *Streptococcus agalactiae* strain ST103 or strain ST12 infected macrophages isolated from the same animals. (PDF 264 kb)

Additional file 7: Table S5. Detailed information on microRNAs significantly differentially expressed between bovine monocyte-derived macrophages infected with *Streptococcus agalactiae* strains ST103 or ST12, and the respective uninfected controls. Padj - Benjamini and Hochberg corrected *P*-value. (XLSX 17 kb)

Additional file 8: Table S6. Biological networks associated with top diseases and functions that were overrepresented among microRNAs differentially expressed between bovine monocyte-derived macrophages infected with *Streptococcus agalactiae* strains ST103 or ST12, and the respective uninfected controls. (XLSX 10 kb)

Additional file 9: Table S7. List of target genes of the differentially expressed miRNA as predicted by TargetScan (cumulative weighted context++ score < – 0.5) in ST103 or ST12 infected bovine macrophages and the list of overrepresented pathways among these target genes as analyzed by InnateDB. (XLSX 410 kb)

Abbreviations

AGO2: Argonaute 2; BAK1: BCL-2 antagonist killer 1; BAX: BCL-2 associated X; BCL2L1: BCL-2 like 1; BMEs: Bovine mammary epithelial cells; CCL11: C-C motif chemokine ligand 11; CCL26: C-C motif chemokine ligand 26;

CFU: Colony-forming units; DE: Differentially expressed; DICER1: Dicer 1, ribonuclease III; GABA: Gamma-aminobutyric acid; GPCRs: G-protein-coupled receptors; IL-10: Interleukin-10; IL-12: Interleukin-12; IL-1β: Interleukin-1 beta; IL-4: Interleukin-4; IL-6: Interleukin-6; IL-8: Interleukin-8; IPA: Ingenuity Pathway Analysis; JaK: Janus kinase; LPS: Lipopolysaccharides; M1: Classically activated macrophages; miRNA: microRNA; miRNome: Complete miRNA content; MOI: Multiplicity of infection; NFKβ: Nuclear factor kappa β; NMBU: Norwegian University of Life Sciences; NR: Norwegian Red; OD: Optical density; PBMC: Peripheral blood mononuclear cells; PCA: Principal component analysis; PPIA: Peptidylprolyl isomerase A; RIN: RNA integrity numbers; RT-qPCR: Reverse transcription-quantitative PCR; SMAD2/3: SMAD family member 2/3; small nucleolar RNAs: snoRNAs; sRNA-seq: Small-RNA-seq; TGFβ1: Transforming growth factor beta 1; TLR: Toll-like receptor; TLR2: Toll-like receptor 2; TNFα: Tumor necrosis factor alpha

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Availability of data and materials
The datasets analyzed during the current study have been deposited in ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-5937.

Authors' contributions
AMLS participated in the design of the study and the RT-qPCR experiment, carried out the infection experiments, analyzed the RT-qPCR and microRNA sequencing data, and drafted the manuscript. SFH participated in the design of the study, cultured the bacteria, carried out infection experiments and performed the RT-qPCR. PB, BH and IO participated in the study design, discussion and interpretation of the results and manuscript drafting. TRS and OØ participated in the study design and discussion of the results. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
Blood sampling was conducted in accordance with the laws and regulations controlling experiments using live animals in Norway and the study was approved by the Norwegian Animal Research Authority (Norwegian Food Safety Authority; FOTS id: 8194).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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