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Short communication: Effect of granulocyte-macrophage colony-stimulating factor on neonatal calf peripheral blood neutrophil function in vitro

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ABSTRACT

Neutrophils are innate immunity cells that represent the first line of cellular defense against invading pathogens. Dairy calves, however, experience neutrophil dysfunction during the first weeks of age, contributing to increased disease susceptibility during this period. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that improves neutrophil function in neonates of other species and mature cows. However, its capability to improve neonatal calf neutrophil function is unknown. Therefore, our objective was to evaluate the effect of GM-CSF on the functional capabilities of neutrophils of neonatal calves in vitro. We hypothesized that supplementation of neonatal neutrophils with GM-CSF would increase microbicidal functions to levels comparable with those of mature immunocompetent cattle. For this, we isolated blood neutrophils from 12 healthy 2- to 3-d-old Holstein calves, and neutrophils from 6 mid-lactation Holstein cows were used as a reference of robust neutrophil function. Subsequently, neutrophils from both calves and cattle were incubated for 9 h with 4 concentrations (0, 0.005, 0.05, or 0.5 μg/mL) of GM-CSF, and microbicidal function of neutrophils was assessed in terms of phagocytosis, respiratory burst, myeloperoxidase (MPO) activity, and extracellular trap formation. Mixed models with Tukey pairwise comparisons were used to identify differences among treatment and age groups. Supplementation of GM-CSF in vitro increased phagocytosis and MPO activity of calf and cow neutrophils, although not in a concentration-dependent manner. Respiratory burst and extracellular trap formation were not affected by GM-CSF supplementation. All the microbicidal capacity functions assessed were lower in neutrophils from calves, but supplementation with GM-CSF increased phagocytosis and MPO activity of calf neutrophils to levels comparable with unsupplemented cow neutrophils. Collectively, our results demonstrated that in vitro supplementation of calf neutrophils with GM-CSF enhanced some functional microbicidal capabilities to levels comparable with immunocompetent cattle. Hence, it may be possible to augment the functional capacity of calf neutrophils in vivo through the therapeutic application of GM-CSF and consequently enhance calves' resistance to infections. This should be tested in future in vivo studies.

Key words: calf health, cytokine, dairy calf, immunomodulation, innate immunity

Short Communication

Calf morbidity and mortality risks associated with infectious diseases have been reported to be high in several countries (NAHMS, 2014; Windeyer et al., 2014; Abuelo et al., 2019). A major factor contributing to disease susceptibility in the neonatal stage is the inability of calves to mount an effective immune response against pathogens (Chase et al., 2008). In addition to the antibodies transferred by colostrum, neonatal calves rely on innate immunity to fight infections (Chase et al., 2008; Cortese, 2009). Neutrophils are immune cells that belong to this innate system and are the first line of cellular defense against invading pathogens. Neutrophils play critical roles in various infectious diseases of cattle (e.g., mastitis, metritis, pneumonia), and decreased neutrophil function has been associated with the development of these diseases (Kehrli et al., 1989; Cai et al., 1994; Hammon et al., 2006; Bassel and Caswell, 2018). In calves, several functions relevant to the microbicidal capacity of neutrophils are decreased during the first few weeks of age (Doré et al., 1990, 1991; Zwahlen and Roth, 1990; Zwahlen et al., 1992; Higuchi et al., 1997; Li et al., 2016), the period when the greatest neonatal morbidity and mortality risks are observed on dairy farms (Windeyer et al., 2014). Specific neutrophil functions relevant to microbicidal capacity altered in neonates include reduced phagocytic ability, respiratory burst activity, and myeloperoxidase (**MPO**) concentration and delayed extracellular trap

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formation (Zwahlen et al., 1992; Chase et al., 2008; Yost et al., 2009).

Colony-stimulating factors (**CSF**) are a group of cytokines that influence functional activity, survival, proliferation, and differentiation of myeloid hemopoietic cells (Nemunaitis, 1997). The administration of granulocyte CSF to dairy cows experiencing periparturient neutrophil dysfunction resulted in neutrophilia and enhanced neutrophil microbicidal capacity, with improvements identified in key microbicidal activities including phagocytosis, respiratory burst, and MPO release (Kehrli et al., 1991; Kimura et al., 2014). Furthermore, improved clinical and production outcomes following peripartum administration of granulocyte CSF have been reported (Canning et al., 2017; Ruiz et al., 2017). However, to our knowledge, the effect of CSF on neonatal calf neutrophil microbicidal capacity remains unexplored. The pathways by which neonatal neutrophils achieve antimicrobial functions differ from those of adult cows (Doré et al., 1990, 1991; Zwahlen and Roth, 1990; Zwahlen et al., 1992; Higuchi et al., 1997; Li et al., 2016). Therefore, the effect of CSF on neonatal neutrophil antimicrobial functions cannot be directly translated from studies in adult cattle. Hence, the objective of this study was to evaluate the effect of CSF supplementation on the functional capabilities of neutrophils of neonatal calves in vitro.

The functional activity of granulocyte-macrophage CSF (**GM-CSF**) is the broadest of the 4 distinct types of CSF (Nemunaitis, 1997), and its prophylactic administration increased survival in a neonatal rat model of infection (Frenck et al., 1990). In mature dairy cows, neutrophils supplemented with GM-CSF in vitro exhibited increased chemotactic and bactericidal capacity (Sordillo et al., 1992). We therefore hypothesized that supplementation of neonatal neutrophils with GM-CSF would increase their microbicidal functions to levels comparable with those of mature immunocompetent cattle.

This study was approved by the Institutional Animal Care and Use Committee of Michigan State University (protocol 2018/002), and animals were used with the owner's consent. Blood was collected from twelve 2- to 3-d-old female Holstein calves from a large commercial dairy farm associated with the Michigan State University Training Center for Dairy Professionals (Elsie, MI). All calves were born from eutocic births with no or little assistance. Immediately after birth, calves received an oral vaccine against diarrhea pathogens (Calf-Guard, Zoetis Services, Parsippany, NJ), an intranasal vaccine against respiratory pathogens (Inforce 3, Zoetis Services), and 3 mL of a vitamin E and selenium complex subcutaneously (MU-SE, Merck Animal Health, Madison, NJ). After 30 min, calves received 4 L of >21% Brix colostrum via orogastric tube and another 2 L of colostrum approximately 6 h later. Calves were housed in pairs in in-house stalls and fed 3 L of milk replacer (Cow's Match ColdFront, Land O'Lakes Inc., Arden Hills, MN) 3 times/d until 7 d of age.

Blood was also collected from 6 mid-lactation (220–250 DIM) Holstein cows in second to fourth lactation to provide an indication of competent neutrophil function because mid-lactation cows are less likely to undergo immune dysfunction (Ingvartsen and Moyes, 2013). Cows were milked thrice daily and were fed a TMR designed to meet or exceed their requirements (NRC, 2001). All animals were clinically healthy in a physical exam conducted before blood sampling.

A total of 50 mL of blood per animal was collected via jugular venipuncture into acid-citrate-dextrose tubes (Becton Dickinson, Franklin Lakes, NJ). Neutrophil isolation started within 40 min after collection following a previously described protocol that relies on Ficoll density-gradient centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes (Kuhns et al., 2015). The isolation procedure yielded a neutrophil population of more than 96% purity as determined using a hematology analyzer (Advia 2120; Siemens Healthcare Diagnostics Inc., Tarrytown, NY), and viability was more than 97% as determined by trypan blue exclusion.

Aliquots of the neutrophils isolated from each animal were resuspended in RPMI medium (Sigma-Aldrich, St. Louis, MO) containing 5% fetal bovine serum (**FBS**; Thermo-Fisher Scientific, Waltham, MA) supplemented with 0.005, 0.05, or 0.5 μg/mL bovine recombinant GM-CSF (Kingfisher Biotech Inc., St. Paul, MN) at a final concentration of 5×10^6 neutrophils/mL. Control neutrophils were resuspended in RPMI medium containing only 5% FBS. Cells were incubated for 9 h at 37°C in 5% CO_2 before assessment of neutrophil function. The incubation time was established based on previous research reporting that the bactericidal activity of adult cattle neutrophils treated with 0.005 μg/mL GM-CSF was not significantly higher than that of control cells until after 9 h of incubation (Sordillo et al., 1992).

Phagocytosis was measured by assessing the engulfment of fluorescein isothiocyanate-conjugated (**FITC**) *Escherichia coli* through flow cytometry using a commercial assay (*E. coli* Phagocytosis Assay Kit, Cayman Chemical, Ann Arbor, MI). Samples were filtered through a 40-μm nylon mesh before measurement by flow cytometry with emission measured at 488 nm (Accuri C6; Becton Dickinson). For each experimental condition, a bacteria-free sample was gated including ≥99% of events and used as a reference. Phagocytosis

in samples incubated with FITC *E. coli* was expressed as the percentage of events beyond that gating that were FITC positive.

The activity of MPO was measured using a fluorescence-based commercial assay (MPO Peroxidation Fluorometric Assay Kit, Cayman Chemical) that relies on the reaction of the hydrogen peroxide generated by MPO activity with 10-acetyl-3,7-dihydroxyphenoxazine to produce the fluorescent compound resorufin. All samples were assayed in duplicate, and the MPO activity is expressed in nanomoles per minute per milliliter.

Respiratory burst was measured based on a published protocol that relies on stimulation of neutrophils to produce superoxide anions via the respiratory burst, which oxidizes luminol and generates an emission detectable via luminescence (Radi et al., 1993; Nemec et al., 2012). In brief, 100 μL of a 2×10^5 neutrophils/mL suspension was prepared in a 96-well chemiluminescence plate (Greiner Bio-One, Monroe, NC). Stimulated samples were produced by addition of 80 μL of phorbol myristate acetate (**PMA**; Sigma-Aldrich) at a final concentration of 160 n*M*, and 80 μL of Hanks' balanced salt solution instead of PMA was added to unstimulated samples. Last, 20 μL of luminol (Sigma-Aldrich) was added to all wells at a final concentration of 10 μ*M*. All samples were analyzed in triplicate. Luminescence was measured every 5 min for 2 h at 37°C. The production of reactive oxygen species was calculated in arbitrary units as the difference between the sum of luminescence measurements over the 2 h in stimulated and corresponding unstimulated samples.

Neutrophil extracellular trap formation was measured based on a standardized protocol that determines the colocalization of DNA and MPO following neutrophil stimulation using flow cytometry (Masuda et al., 2017). Briefly, 1×10^6 neutrophils were suspended in 1 mL of RPMI medium + 5% FBS and preincubated for 30 min. Stimulated samples were prepared by adding PMA to a final concentration of 100 n*M* and incubating for 1 h at 37°C, whereas 500 μL of Hanks' balanced salt solution was added to nonstimulated samples. Neutrophils were then washed with PBS. To block nonspecific binding of antibody, neutrophils were incubated for 30 min in 100 μL of Tris-buffered saline and 0.5% Tween 20 (Genemed, Torrance, CA) containing 5% normal goat serum (ImmunoReagents Inc., Raleigh, NC) at a concentration of 1×10^7 cells/mL. Neutrophils were then incubated in 5 μg/mL anti-human MPO antibody conjugated to phycoerythrin [MPO monoclonal antibody (MPO455-8E6), PE, eBioscience; cat. no. 12-1299-42; Thermo-Fisher Scientific] for 30 min at room temperature. Neutrophils were then washed and resuspended in 1 mL of PBS and subsequently stained with DNA-binding SYTOX Green (Thermo-Fisher Scientific). After filtering through a 40-μm nylon mesh, the cells were analyzed using flow cytometry with emission measured at 523 nm for SYTOX Green and 576 nm for phycoerythrin (BD Accuri C6; Becton Dickinson). For each experimental condition, events measured by both emission wavelengths were plotted against each other. The unstimulated sample of each experimental condition was gated and used as a reference for basal fluorescence of unstimulated neutrophils. Extracellular trap formation in samples incubated in PMA was expressed as the percentage of neutrophils beyond that gating that were positive to both emission wavelengths.

Statistical analysis was conducted in JMP Pro 14 (SAS Institute Inc., Cary, NC). Mixed models were built for the outcome variables respiratory burst, phagocytosis, MPO, and extracellular trap formation. The fixed effects included age (calf vs. cow), treatment (0, 0.005, 0.05, or 0.5 μg/mL GM-CSF), and age × treatment interaction. Each animal was included as a random effect to account for individual variability. Tukey's honest significance test was used for post hoc pairwise comparisons. To ensure homoscedasticity of residuals, the data of respiratory burst and MPO were log-transformed. Data are presented as least squares means (95% confidence intervals). Statistical significance was declared at $P < 0.05$.

Treatment with GM-CSF in vitro increased the ability of neonatal calf and mature cow neutrophils to phagocyte *E. coli* (Figure 1A). The effect observed was independent of the concentration of GM-CSF used, with similar phagocytic abilities among the different GM-CSF concentrations within each age group. Untreated neonatal neutrophils exhibited lower phagocytosis capacity than untreated neutrophils isolated from immunocompetent mid-lactation cattle (25.1 vs. 36.3%; $P = 0.009$). However, when treated with GM-CSF, the proportion of neonatal neutrophils showing phagocytic ability increased to levels similar to those of untreated ($P > 0.443$) and GM-CSF–treated ($P > 0.154$) neutrophils from mature cattle.

Similarly, the activity of MPO increased in GM-CSF–treated neutrophils of both calves and cows compared with untreated neutrophils in a non-dose-dependent manner (Figure 1B). In the untreated neutrophils, the activity of MPO was lower in calves compared with cows ($P = 0.0001$). Nevertheless, supplementation with GM-CSF increased MPO activity of neonatal neutrophils to a level similar to that of untreated neutrophils from mature cows ($P > 0.136$). This increase, however, was not sufficient to reach the MPO activity of GM-CSF–treated neutrophils from adult cattle ($P \leq 0.003$).

On the other hand, in vitro treatment with GM-CSF for 9 h at the studied concentrations did not influence the respiratory burst (Figure 1C; $P = 0.644$) or extra-

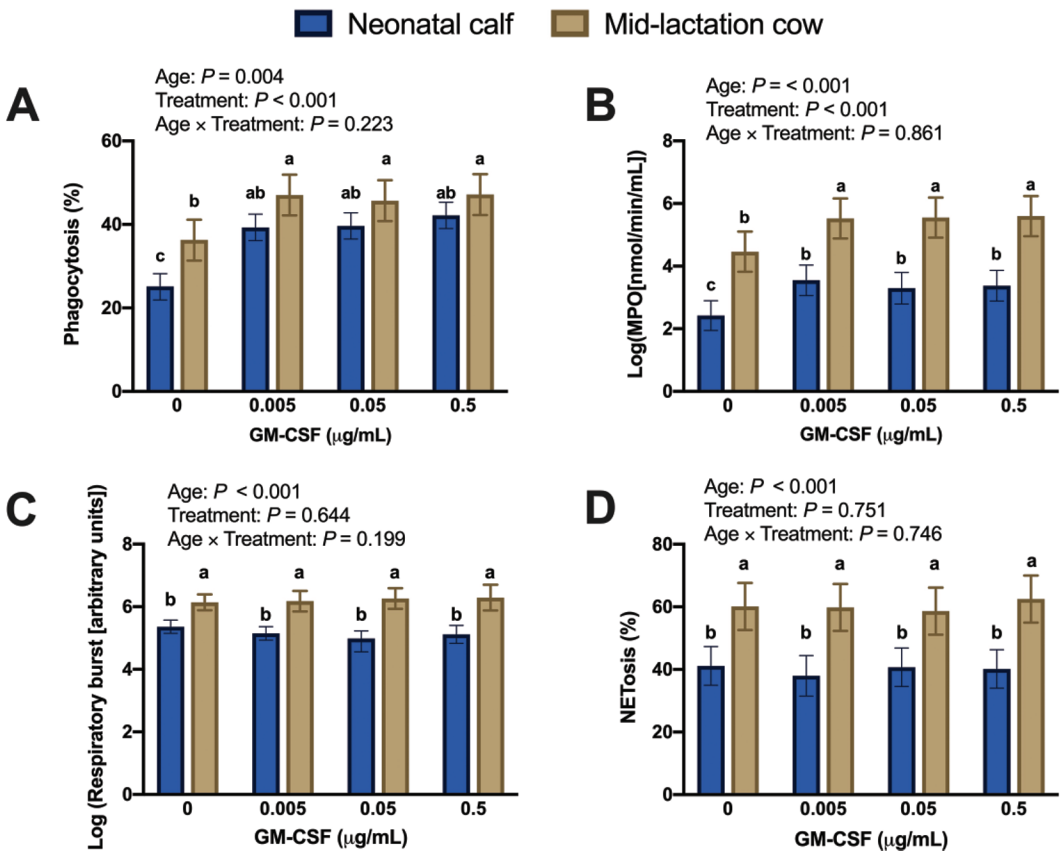

Figure 1. Effects of increasing doses of granulocyte-macrophage colony-stimulating factor (GM-CSF) on the (A) phagocytic capabilities, (B) myeloperoxidase (MPO) activity, (C) respiratory burst, and (D) extracellular trap formation (NETosis) of neutrophils obtained from blood of 2- to 3-d-old dairy calves or mid-lactation dairy cows. The neutrophils were incubated for 9 h with 0, 0.005, 0.05, or 0.5 μg/mL bovine recombinant GM-CSF. Data were analyzed via mixed models with the fixed effects of age group (calf vs. cow), treatment (concentrations of GM-CSF), and age × treatment interaction. Animal was included as a random effect. Data of MPO and respiratory burst were log-transformed to meet normal distribution of residuals. Results are expressed as least squares means (logarithm) and 95% CI. Bars with different letters (a–c) are significantly different ($P < 0.05$).

cellular trap formation capacity (Figure 1D; $P = 0.751$) of either neonatal calf or mature cow neutrophils. In both function assays, neutrophils isolated from neonatal calves showed lower capabilities compared with those isolated from immunocompetent adult cattle ($P < 0.001$).

Diseases of neonatal dairy calves have substantial negative consequences for the dairy industry, including economic costs and animal welfare issues (Donovan et al., 1998). Management practices have improved on dairy farms, yet calf morbidity and mortality remain above ideal standards in many countries (NAHMS, 2014; Windeyer et al., 2014; Abuelo et al., 2019). It is likely that the extensive immune dysfunction exhibited by neonatal dairy calves contributes significantly to disease susceptibility (Chase et al., 2008). Neutrophils provide the first line of defense of the innate immune system. Neutrophils eliminate the pathogens by phagocytosis as well as by forming extracellular traps, which comprise DNA decorated with histones and granular proteins (Kaplan and Radic, 2012). Subsequently, neutrophils kill bacteria via the production of reactive oxygen species generated in the respiratory burst and by oxidized halides produced by MPO (Weiss and Walcheck, 2008). In neonatal calves, several functions relevant to the capacity of neutrophils to kill pathogens are decreased during the first few weeks of age (Doré et al., 1990, 1991; Zwahlen and Roth, 1990; Zwahlen et al., 1992; Higuchi et al., 1997; Li et al., 2016). Despite this critical role in the immune response initiation, research on methods to improve the neutrophil function of newborn calves has been limited. In the current study, we explored the in vitro effect of GM-CSF on functions relevant to microbicidal capacity of neonatal calf neutrophils. Neutrophils from mid-lactation adult cattle were used to provide a reference of these functions in healthy, immunocompetent animals. In this study, we focused on the neutrophil function of newborn (2- to

3-d-old) calves because the first few days of life are an important risk period for diarrhea in these animals (Windeyer et al., 2014). However, maternal immune cells from colostrum are likely also present in the blood of these calves (Reber et al., 2006); therefore, it is likely that the isolated neutrophils are a mixture of calf- and colostrum-derived cells.

Our results show that neutrophils from calves have a lower capacity to phagocyte *E. coli* than those isolated from mature cattle. This reduced phagocytic ability has been previously reported (Menge et al., 1998); however, supplementation with GM-CSF increased the percentage of phagocytizing neutrophils to levels similar to those of immunocompetent cattle. The effect of CSF in enhancing phagocytosis in mature neutrophils has already been reported in both humans and cattle (Fleischmann et al., 1986; Kehrli et al., 1991). To our knowledge, this is the first study exploring the effect of GM-CSF in neutrophils from newborn calves, but a similar enhancement of phagocytosis was also observed in infants (Ahmad et al., 2004). The changes to phagocytic activity observed in this in vitro study must be confirmed by in vivo trials because neutrophil activation varies with stimulant, stimulant dose, stimulant interactions, and other signaling factors (Witko-Sarsat et al., 2000), all of which vary under field conditions on dairy farms.

The release of MPO is one of the central mechanisms by which adult neutrophils kill invading pathogens (Klebanoff et al., 2013). In line with previous research (Weiss and Walcheck, 2008), the MPO activity was lower in neutrophils from neonatal calves compared with adult cattle. Supplementation of GM-CSF in vitro increased the MPO activity in blood neutrophils from both adult cattle and neonatal calves. The positive effect of GM-CSF on neutrophil activity of MPO has been previously reported in both human adults and children (Dang et al., 1993; Liu et al., 2018). In dairy cattle, peripartum administration of granulocyte CSF, a cytokine from the same family, also resulted in increased MPO release from neutrophils (Kimura et al., 2014). Interestingly, no concentration-dependent effect of GM-CSF on phagocytosis or MPO activity was detected. Sordillo et al. (1992) investigated the effect of increasing concentrations of GM-CSF on blood and mammary gland neutrophils from adult cattle. They also found an increase in bactericidal activity that was not proportional to concentration, thus suggesting that there may be a threshold of immunomodulation of GM-CSF on neutrophils from neonate and adult bovines.

Supplementation with GM-CSF did not improve respiratory burst or extracellular trap formation in adult or neonatal neutrophils compared with untreated neutrophils. Although the respiratory burst response of human neutrophils improved with GM-CSF (Jaeger et al., 1999), no effect of CSF on respiratory burst was detected in neutrophils from periparturient cattle (Kehrli et al., 1991; Kimura et al., 2014). These changes could be associated with differences in signal-transduction pathways that regulate the effect of GM-CSF priming on the respiratory burst activation (Leino et al., 1993). A reduction in extracellular traps of neonatal compared with mature neutrophils was also noted (Figure 1D). To our knowledge, this is the first study comparing extracellular trap formation between neonatal and adult cattle neutrophils. However, a similar reduction in trap has been observed in human infants (Marcos et al., 2009; Yost et al., 2009). Similarly, little information is available on the effect of GM-CSF on extracellular trap formation, although one study in humans showed enhanced formation with simultaneous GM-CSF priming and complement factor 5a stimulation (Yousefi et al., 2009). Hence, the capacity of GM-CSF to improve trap formation in neonatal neutrophils needs to be further explored using other stimulants. Additionally, the flow cytometry protocol used does not differentiate between soluble MPO colocalized with the traps and neutrophil surface expression of MPO. Hence, microscopy could be used to better understand the effect of CSF on the extracellular trap formation capacity of newborn calf neutrophils.

The therapeutic use of granulocyte CSF in dairy cattle experiencing periparturient neutrophil dysfunction resulted in neutrophilia and enhancement of some neutrophil microbicidal functions (Kimura et al., 2014; Van Schyndel et al., 2018) as well as upregulation of genes involved in migration, pattern recognition, and inflammatory response ability (Heiser et al., 2018). Despite these reported improvements in neutrophil functionality, results in the literature about the effectiveness of the periparturient prophylactic use of granulocyte CSF to reduce disease incidence and severity are mixed. Some studies reported reduced severity and susceptibility to mastitis (Canning et al., 2017; Ruiz et al., 2017; Powell et al., 2018) and a lower incidence of retained placenta (Ruiz et al., 2017), but increased incidence of metritis and displaced abomasum has also been reported (Ruiz et al., 2017; Zinicola et al., 2018). Hence, although GM-CSF resulted in improved neonatal neutrophil function in vitro, further studies in calves are needed to elucidate whether this results in reduced neonatal calf morbidity. Granulocyte CSF and GM-CSF belong to the same family of cytokines and have similar functions, but it cannot be assumed that both cytokines will elicit the same effects. Therefore, further studies should also examine the potential effect of granulocyte CSF therapy in newborn calves. Similarly, given the clearance of maternal immune cells from the calf's bloodstream and

the continuous maturation of the calf's own immune cells, it will be necessary to establish the optimal age for colony stimulation therapy in newborn calves. Also, the lack of a dose-dependent response of the GM-CSF treatment observed in this study has potential implications for future in vivo studies, as maintaining the exposure to GM-CSF for a longer time might be more important than increasing its concentration once the threshold of activity is achieved.

Collectively, our results showed that in vitro supplementation of neonatal neutrophils with GM-CSF enhanced some microbicidal capabilities to levels comparable with those of immunocompetent mature cattle. Thus, the therapeutic application of CSF could potentially be used to enhance neutrophil function in calves and subsequently reduce neonatal morbidity and mortality. Further studies are needed to evaluate these effects in vivo.

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