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Postnatal changes in epigenetic modifications of neutrophils of foals are associated with increased ROS function and regulation of neutrophil function

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ABSTRACT

Neonates of all species, including foals, are highly susceptible to infection, and neutrophils play a crucial role in innate immunity to infection. Evidence exists that neutrophils of neonatal foals are functionally deficient during the first weeks of life, including expression of cytokine genes such as *IFNG*. We hypothesized that postnatal epigenetic changes were likely to regulate the observed age-related changes in foal neutrophils. Using ChIP-Seq, we identified significant differences in trimethylated histone H3 lysine 4, an epigenetic modification associated with active promoters and enhancers, in neutrophils in foals at 30 days of age relative to 1 day of age. These chromatin changes were associated with genes implicated in immune responses and were consistent with age-related changes in neutrophil functional responses including ROS generation and IFN expression. Postnatal changes in epigenetic modifications suggest that environmentally-mediated cues help to promote maturation of neutrophil functional responses. Elucidating the environmental triggers and their signaling pathways could provide a means for improving innate immune responses of neonates to improve their ability to combat infectious diseases.

1. Introduction

Neonatal mammals are highly susceptible to infectious diseases because they are immunologically naïve and immature (Adkins et al., 2004; Basha et al., 2014; Cuenca et al., 2013). The lag required for adaptive immune responses to occur and to develop to full potential renders neonates highly dependent on innate immune responses for protection against early infections (Adkins et al., 2004; Basha et al., 2014; Cuenca et al., 2013; Maródi, 2006). Neutrophils are the most abundant innate immune cells in circulation and are the first cells to respond to infection (Cuenca et al., 2013; Lawrence et al., 2017; Maródi, 2006). Moreover, they secrete effector molecules in response to infection that help direct adaptive immune responses (Appelberg, 2006; Lawrence et al., 2017; Sawant and McMurray, 2007; Tachhini-Cottier et al., 2000). Despite their key role in neonatal defense, evidence exists that functional responses of neutrophils of newborn animals, including foals, differ from those of more mature animals (Appelberg, 2006; Bordin et al., 2012; Cohen et al., 2014; Cuenca et al., 2013; Lawrence et al., 2017; Maródi, 2006; Nerren et al., 2009; Sawant and McMurray, 2007; Tachhini-Cottier et al., 2000).

Although the precise mechanisms regulating these developmental changes in neutrophil function are unknown, several lines of evidence indicate that epigenetic mechanisms may play a role. First, Th-cell lineage responses are known to change with age (Adkins et al., 2004; Basha et al., 2014), and there is compelling evidence that T-cell lineage differentiation is regulated epigenetically (Wilson et al., 2009). Second, epigenetic regulation of neutrophil genes has been reported under physiological conditions (Jelinek et al., 2007). Third, evidence exists that transcription factors, including those in the NF-κB and C/EBP families, regulate not only neutrophil differentiation from myeloid precursors but also cytokine expression in mature neutrophils, and these transcription factors may be influenced by epigenetic modifications (Cloutier et al., 2009). Fourth, the *IFNG* promoter of foal lymphocytes has been observed to be hypermethylated relative to adults, and age-

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associated demethylation was correlated with increased expression of interferon-γ (IFN-γ) (Sun et al., 2013).

To our knowledge, evaluation of postnatal changes in epigenetic modifications in neutrophils of neonates has not been investigated. Here, we show that changes in epigenetic modifications that occurred during the first month of life were located at genes associated with neutrophil maturation and function, and that these modifications corresponded with an increase in *functional* response of foal neutrophils. Specifically, we examined reactive oxygen species (ROS) production by neutrophils obtained sequentially at ages 1 day and 30 days and performed chromatin immunoprecipitation (ChIP) coupled with massively parallel sequencing (ChIP-Seq) to identify genomic regions with specific histone modifications, *viz.*, trimethylated histone H3 lysine 4 (H3K4me3), a mark of active gene transcription, and trimethylated histone H3 lysine 27 (H3K27me3), a mark of repressed transcription (Allis and Jenuwein., 2016; ENCODE Project Consortium, 2012). These results provide a first map of these histone modifications in equine neutrophils and identify key regulators of neutrophil functional deficits in foals.

2. Materials & methods

2.1. Foals

The protocol for this study was approved by the Institutional Animal Care and Use Committee at Texas A&M University (AUP# 2010-002). Five healthy Quarter Horse foals born at the Texas A&M University Horse Center were used for this study. All foals received adequate transfer of colostral immunoglobulins as determined by a commercial immunoassay (SNAP Test, IDEXX, Inc) and were deemed healthy on the basis of physical inspection and results of a complete blood count performed on day 1 of life. Approximately 56 ml of blood were collected from foals on the day of birth and 30 days (30 days) later, using 20% citrate dextrose solution as an anticoagulant.

2.2. Neutrophil isolation

For neutrophil isolation, whole blood was allowed to settle at room temperature for 30–45 min, and then the plasma and buffy coat layers were used for neutrophil purification as described previously (Cohen et al., 2014). Neutrophils were isolated by layering whole blood over a discontinuous Histopaque (Sigma-Aldrich, St. Louis, MO, USA) gradient (specific gravity 1.077 over specific gravity 1.119) and centrifuged (700 × g, 30 min, room temperature) (Kjeldsen et al., 1999). After centrifugation, the 1.119 band containing the neutrophils was removed and washed once with Hanks balanced salt solution (HBSS) and centrifuged (3006 × g, 10 min). The neutrophil concentration was determined using an automated cell counter (Cellometer Auto T4, Nexcelcom, Lawrence, MA, USA), and purified neutrophils were examined microscopically to determine purity (to confirm > 98% of neutrophils in the cell preparations). Neutrophils then were resuspended in HBSS to a final concentration of 2×10^7 cells/ml.

2.3. Detection of neutrophil ROS

Neutrophils were suspended in 1 ml HBSS and kept on ice until aliquoted for the ROS assay. A 10 mg/ml stock solution of dihydrorhodamine-123 (DHR; Sigma-Aldrich, St. Louis, MO, USA) was prepared in DMSO and stored at −20 °C. This solution was diluted in HBSS to a 25 mg/ml working solution and stored in the dark until use. For ROS production, neutrophils (1×10^6 cells) were incubated at 37 °C with 2.5 mg DHR alone (negative control) or 16 ng of phorbol 12-myrisate 13-acetate (PMA) as stimulus (positive control). The number of reactive neutrophils and mean fluorescent intensity was analyzed using a FACScan flow cytometer equipped with a 488-mm argon laser and Cell Quest Analysis Software (Beckton Dickinson, San Jose, CA, USA). A total of 10,000 data events were collected. Outcomes quantified included the number of reactive neutrophils, the proportion of reactive neutrophils, and the product of the mean fluorescence intensity and the proportion of reactive neutrophils (*i.e.*, mean fluorescence intensity x proportion of reactive neutrophils). Linear mixed-effects modeling was used for analysis of ROS data, with individual foal modeled as a random effect to account for repeated measures over time (ages), stimulus modeled as a categorical variable (with negative control as the reference group) and age modeled as a fixed, ordered categorical effect. Post hoc testing among times or stimuli was performed using the method of Sidak. A significance level of $P < 0.05$ was used for all statistical analyses, which were performed using S-PLUS (version 8.2) statistical software (TIBCO, Seattle, WA, USA).

2.4. Chromatin isolation

Chromatin was isolated from neutrophils using a modified nitrogen cavitation protocol [18]. Briefly, purified neutrophils (2.5×10^7 cells/mL) from each animal at each developmental time point were resuspended in relaxation buffer and then transferred to the nitrogen apparatus. The apparatus was pressurized with nitrogen (450 psi, 15 min, room temperature), and then the pressure was relieved through the discharge valve. The supernatant was removed from the apparatus, transferred to a sterile tube, and then centrifuged (1500 × g, 10 min, 4 °C) to pellet the nuclei. The supernatant was decanted and nuclei resuspended in MNase Digestion Buffer (0.32 M sucrose, 0 mM Tris-HCl, 4 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl flouride). Twenty units of MNase (Thermo Fisher Scientific, Waltham, MA, USA) was then added to each sample and incubated (37 °C, 10 min). The reaction was stopped with EDTA (50 μM). Two samples of each animal at each time point were processed and then pooled after the chromatin was isolated.

2.5. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed to identify regions enriched with trimethylated histone H3 lysine 4 (H3K4me3) or trimethylated histone H3 lysine 27 (H3K27me3) as previously described (Dindot et al., 2009) but with slight modifications. Briefly, H3K4me3 (Catalog # 17-614) and H3K27me3 (Catalog # 17-622) primary antibodies (Millipore, Burlington, MA USA) were incubated as independent immunoprecipitation reactions with digested chromatin and rotated (12 h, 4 °C). Dynabeads (Invitrogen) conjugated with mouse-anti-IgG antibody were then added to each reaction and rotated (2 h, 4 °C). The samples were washed 6-times (1X, low salt buffer: 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl; 2X, high salt buffer: 0.1% SDS, 1% Triton X-100, 500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl; 1X, LiCl buffer: 0.25 M liCl, 1% NP-40, 1% Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, Complete Protease Inhibitor; 2X Tris-EDTA buffer: 10 mM Tris, 0.5 M EDTA) and then the ChIP DNA was eluted (1 M $NaCHO_3$, 20% SDS). DNA was purified from the chromatin using a MinElute Purification Column (Qiagen, Germantown, MD).

2.6. ChIP-sequencing

Sequencing libraries were constructed for each sample using a standard ChIP-Seq protocol (Illumina, San Diego CA). Briefly, 10 ng of DNA was blunt end-repaired, adenylated, and ligated with single-end adaptors, according to the manufacturer's protocol (Illumina). The prepared library was resolved on a 2% low-range agarose gel and a section of corresponding to 175-250 base-pairs (bp) of DNA was extracted (Qiagen). The library was then enriched according to the manufacturer's protocol (Illumina). The sequencing library size was determined using polymerase chain reaction (PCR), polyacrylamide gel electrophoresis (PAGE) and the Agilent 2100 Bioanalyzer (Agilent Technologies, San Diego CA). Single-end 36 nucleotide, massively-