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Research paper

Epigenetic contribution to individual variation in response to lipopolysaccharide in bovine dermal fibroblasts

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ABSTRACT

The innate immune signaling pathway plays a crucial role in the recognition and early response to pathogens associated with disease. Genetic analysis has been unable to completely account for individual variability in the strength of the innate immune response. The aim of this study was to determine the role of the epigenetic markers (DNA methylation or histone acetylation) in controlling bovine gene expression in relation to the response to lipopolysaccharide (LPS). To determine the impact epigenetics may have in controlling innate immunity, dermal fibroblasts from fifteen dairy heifers having previously displayed a differential response to LPS were exposed to 5-aza-2′-deoxycytidine (AZA) and trichostatin A (TSA); de-methylating and hyper-acetylating agents, respectively. The AZA–TSA exposure resulted in a loss of variability between individuals' response to LPS as measured by fibroblast IL-8 protein production. Transcriptomic analysis by microarray was used to elucidate the role of epigenetics in innate immune signaling at 2, 4, and 8 h post-LPS exposure. A subset of genes displayed altered expression due to AZA–TSA alone, suggesting an epigenetic regulatory element modifying expression under normal conditions. Treatment with AZA–TSA also led to increased expression of IL-8 (7.0-fold), IL-6 (2.5-fold), TNF-α (1.6-fold), and serum amyloid A 3 (SAA3) (11.3-fold) among other genes compared to control cultures for at least one of the measured times following LPS exposure. These data support the conclusion that epigenetic regulation significantly alters LPS-induced responses and constitutive cytokine gene expression.

1. Introduction

There is a growing body of evidence to suggest that components of the adult immune response are established very early in life. The developmental origins of health and disease hypothesis suggest that environmental stimuli present following conception and until birth may play a role in increased rates of disease (Gluckman et al., 2005). The fetal environment is closely associated with maternal status during pregnancy, and differences in maternal conditions have been associated with the development of diabetes, hypertension, and asthma in humans (Bousquet et al., 2004; Gluckman et al., 2005). Variation in the intrauterine environment throughout pregnancy may thus play a large role in determining the phenotype of the offspring. A proposed mechanism for environmental modulation of the immune response is through alteration of epigenetic markers important in controlling gene expression. Epigenetic effects are regulated through DNA methylation and histone acetylation that affect transcription factor access to DNA through chemical modification of DNA binding sites and alterations in chromatin structure, respectively (Bogdanović and Veenstra, 2009; Sawan and Herceg, 2010). For example, variation in methylation status of the *interleukin(IL)-6* and *IL-8* gene promoters in human cell models investigating periodontitis and rheumatoid

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arthritis appear to predispose some subjects to chronic inflammation through a hyper-responsiveness phenotype (Andia et al., 2010; Ishida et al., 2011). In addition to this, increasing histone acetylation in human intestinal epithelial cells (IEC) was able to potentiate the cellular response to lipopolysaccharide (LPS) as measured by IL-8 protein production (Angrisano et al., 2010). Components of the pathogen detection and signaling pathways mediating the response to LPS have also been implicated as regions susceptible to epigenetic control. Epigenetic suppression, mediated by DNA-methylation, of *toll-like receptor (TLR)-4* gene expression has been linked to a lowered response to LPS in intestinal epithelial cells (Takahashi et al., 2009). Conversely, DNA hypomethylation has been implicated in over expression of *myeloid differentiation factor (MD)-2* in human IECs leading to higher responsiveness to LPS exposure (Vamadevan et al., 2010). These findings suggest that methylation and acetylation may play an important role in the regulation of immune-responsive genes involved in pathogen recognition and subsequent signaling. Investigation of the role of epigenetic variation between individuals in modifying their immune response capability would benefit our understanding of human and animal health.

Studies conducted on pregnant rats have shown that prenatal exposure to LPS leads to a suppressed innate immune response in offspring when examined at 5 days post birth (Hodyl et al., 2008) or even after 40 weeks of life (Williams et al., 2011). Considering the ability of the maternal environment to influence the adult immune response (through epigenetic modulation), variation in the intrauterine environment may play a major role in causing individual variation in susceptibility to disease. The dairy cow is one animal for which maternal environment is not uncommonly associated with metabolic or infectious disease. A goal of dairy production is to ensure that dairy cows in their second or greater lactations are also pregnant. Mastitis and other systemic infections are not uncommon occurrences during a dairy cow's pregnancy and may affect the phenotypic response to pathogens exhibited by her offspring. Interestingly, dairy cows exhibit a range of responses to experimental mastitis challenge and yet traits associated with mastitis such as milk somatic cell count and incidence of clinical mastitis have very low heritability (Dal Zotto et al., 2007), suggesting only a minor genetic influence. However, variation experienced while in utero could have epigenetic consequences that may predispose some animals to having an impaired innate immune response leading to reduced health and less profitability for the producer, and limiting the accuracy of genetic selection for mastitis resistance.

Our hypothesis is that a large degree of between-animal variation in the innate immune response of dermal fibroblasts obtained from groups of calves or cows (Green et al., 2011; Kandasamy et al., 2011) is due to epigenetic variation. We aimed to investigate this through in vitro manipulation of cellular DNA methylation and histone acetylation. Modulation of epigenetic markers was accomplished using the chemical inhibitors 5-aza-2′-deoxycytidine (AZA) and trichostatin A (TSA) that inhibit DNA methyltransferase (DNMT) and histone deacetyltransferase (HDAC), respectively. This treatment effectively reprograms the epigenetic makeup of the fibroblasts and removes animal-to-animal variation in epigenetic status. We subsequently compared the ability of cells treated with or without AZA, and with or without TSA, to recognize and respond to LPS. As well, we used genomic expression arrays to identify immune-responsive genes affected by the epigenetic modification. Our results indicate that DNA methylation and histone acetylation are major causes of individual variation observed in the innate immune response of bovine dermal fibroblasts.

2. Materials and methods

2.1. Dermal fibroblast cultures

All experiments were performed with approval of the Institutional Animal Care and Use Committee at the University of Vermont. Primary dermal fibroblast cultures were selected from a collection of cultures obtained from 15 sixteen-month old Holstein heifers for which the fibroblast collection, isolation, and ability to produce IL-8 in response to LPS has previously been described (Green et al., 2011). Fibroblast cultures were selected based on the production of IL-8 in response to LPS exposure, with the three lowest responding (LR) and three highest (HR) responding cultures chosen for further investigation.

2.2. Fibroblast epigenetic treatment and LPS challenge

Following recovery from cryopreservation and expansion in a T-75 cm^2 flask, cells were trypsinized and seeded in 6-well culture plates at 3.0×10^4 cells/mL in a total volume of 2 mL. Cells undergoing epigenetic treatment were either exposed to 5-aza-2′-deoxycytidine (AZA; Sigma), trichostatin A (TSA; Sigma) or a combination of the two to achieve DNA demethylation, histone hyperacetylation, or both, respectively. Cells undergoing DNA demethylation were cultured for 96 h in the presence of 10 μM AZA. Cells selected to undergo histone hyperacetylation were cultured for 72 h in plain medium at which point 80 nM TSA was added for 24 h. Finally, for cells undergoing both treatments, 10 μM AZA was added again for 96 h. At the 72-h time point, 80 nM TSA was included for the final 24 h. Control cells were grown for 4 days with comparable amounts of the AZA–TSA diluents (PBS and DMSO, respectively) added. Dosages and exposure time to AZA and TSA were modeled after previous experiments conducted that displayed low cytotoxicity from treatment in conjunction with effective epigenetic remodeling at similar or lower concentrations than those used in our trials (Duijkers et al., 2013; Takahashi et al., 2009; Tsai et al., 2012).

Following epigenetic modification, cells were washed 3× with PBS and exposed to LPS for 24 h. LPS treatment consisted of growth media supplemented with 100 ng/mL of ultra-pure LPS isolated from *Escherichia coli* O111.B4 (Sigma-Aldrich). Following appropriate exposure time, media was removed, spun at $500 \times g$ for 5 min to remove cell debris, and immediately stored at −20 °C until further analysis.