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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 hours 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. This data supports the conclusion that epigenetic regulation significantly alters LPS-induced responses and constitutive cytokine gene expression.

Keywords

TLR4; methylation; acetylation; innate immunity

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

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6. AUTHOR CONTRIBUTIONS

BG drafted the manuscript, performed the cell culture, the ELISA, the RT-PCR and RNA prep for microarray. DK made substantial contributions to concept and design and critical edits of the manuscript.

5. COMPETING INTERESTS

The authors declare that they have no competing interests.

hypothesis suggests 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 (Bogdanovi 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 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 (HAT) 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 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-75cm² 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 hours in the presence of 10 μ M AZA. Cells selected to undergo histone hyperacetylation were cultured for 72 hours in plain medium at which point 80 nM TSA was added for 24 hours. Finally, for cells undergoing both treatments, 10 μ M AZA was added again for 96 hours. At the 72-hour time point, 80 nM TSA was included for the final 24 hours. 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 3X with PBS and exposed to LPS for 24 hours. 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 x g for 5 minutes to remove cell debris, and immediately stored at -20°C until further analysis.

2.3 Quantification of IL-8

The concentration of IL-8 in fibroblast conditioned media was quantified by a custom sandwich ELISA previously described (Kandasamy et al., 2011) using mouse anti-bovine IL-8 monoclonal antibody (clone 170.13 generously gifted by S. Maheswaren, University of Minnesota) and biotinylated goat anti-human IL-8 antibody (R&D Systems Inc., Minneapolis, MN) as capture and detection antibodies, respectively, and recombinant bovine IL-8 (Thermo Scientific, Rockford, IL) as assay standard. The detection limit of the assay was approximately 300 pg/ml.

2.4 Gene Expression Analysis Following AZA-TSA Treatment

The three fibroblast cultures with the lowest response to LPS treatment were selected for genome-wide expression analysis following AZA-TSA treatment using the Affymetrix custom bovine GeneChip array version1.0 (Affymetrix, Santa Clara, CA). This platform consists of 24,342 gene-level probe sets without replicates. Poly-A controls used were *dap*, *lys*, *phe*, and *thr* while hybridization controls were *bioB*, *bioC*, *bioD*, and *creX*. Following recovery from cryopreservation, cells were expanded and seeded into 6-well plates and kept under control conditions or treated with AZA and TSA as previously described. Fibroblasts were then exposed to LPS (100 ng/mL) for 0, 2, 4, or 8 hours, after which cell lysates were prepared for RNA isolation using a PurePerfect Kit (5 Prime, Gaithersburg, MD). On-column DNase digestion was performed to remove DNA during the RNA isolation procedure. Quality and quantity of the isolated RNA samples was determined with a 2100 Bioanalyzer (Agilent). Subsequently, gene expression analysis was conducted at the University of Vermont's core microarray facility. A total of 24 microarray slides were probed using RNA from three different LR animals. For each animal, sets of four paired cultures were either pre-exposed to AZATSA (as above) or held under control conditions for an equivalent length of time. After exposure to LPS (100 ng/ml) for 0, 2, 4, or 8 hours, RNA was collected for transcriptome analysis (GEO accession number GSE50405). Hybridization mixes were prepared and hybridized to the arrays as per manufacturer's recommendations using the Affymetrix 450 fluidics station and scanned with the Affymetrix 3000-G7 scanner.

2.5 Microarray Statistical Analysis

Probe statistics (CEL files) were calculated from scanned images using Affymetrix GCOS software. All other calculations were performed using Partek Genomics Suite tools (Partek, Inc., St. Louis, MO). Probe set and sample matrix expression statistics were calculated using the Robust Multichip Average (RMA) method (Irizarry et al., 2003). Sample quality was assessed based on the 3':5' ratio, relative log expression (RLE), and normalized unscaled standard error (NUSE). Principal Component Analysis (PCA) was also used to look for outlier samples that would potentially introduce latent variation into the analysis of

differential expression across sample groups. Linear modeling of sample groups was performed using ANOVA as implemented in Partek Genomic Suites. The magnitude of the response (fold change calculated using the least square mean) and the p-value associated with each probe set and binary comparison were calculated, as well as a step-up, adjusted p-value for the purpose of controlling the false discovery rate (FDR).

When analyzing the effects of AZA-TSA on the cultures, genes were considered differentially expressed in comparison to controls if they passed the $FDR < 0.01$ and fold-change 1.5 thresholds. To determine the effects of LPS on gene expression, cultures at 2, 4, and 8 hour time points were compared to 0h cultures. Genes were considered differentially expressed if they passed the $FDR < 0.05$ and fold-change 1.5 thresholds. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for functional annotation and analysis by uploading Affymetrix-based nomenclature of statistically significant genes (1.5-fold expression cut-off; $FDR < 0.05$; <http://david.abcc.ncifcrf.gov>).

2.6 Quantitative Real-Time PCR

A subset of genes identified by microarray analysis was selected for expression analysis by quantitative real-time PCR (RT-PCR) using oligonucleotide primers specific to *TLR4*, *IL-8*, *IL-6* (Pareek et al., 2005), *serum amyloid-A (SAA)-3*, *TNF- α* , and *CC-motif ligand (CCL) 20* (Table 1). The same RNA samples used in the genome-wide expression study were used. First-strand cDNA synthesis was conducted using the Improm II Reverse Transcriptase Kit (Promega). Messenger RNA expression was quantified by RT-PCR with a CFX96 Real-Time Instrument (Bio-Rad, Hercules, CA) using PerfeCTa SYBR Green Super-Mix, Low ROX kit (Quanta Biosciences). Cycling conditions were: initial denaturation at 95° C for 2 minutes; then 40 cycles consisting of denaturation at 95° C for 15 seconds, annealing at 60° C for 30 seconds and extension at 72° C for 1 minute. Melt curve analysis was also performed to check amplification of the desired gene product. The *β -actin* gene was used as reference gene for normalization procedure. Threshold cycles (Ct) generated using RT-PCR experiment for each sample were analyzed using the delta Ct method.

2.7 Statistical Analysis

IL-8 protein production was analyzed between groups (LR vs. HR) using a Student's t-test (Graph Pad Prism 5.0). Analysis of real time gene expression data was conducted using a two-way ANOVA model with repeated measures (Graph Pad Prism 5.0). Comparisons with $P < 0.05$ were considered statistically significant within experiments.

3. RESULTS

3.1 Response to LPS Following Epigenetic Modification

IL-8 was undetectable prior to the addition of LPS in both the control and AZA-TSA cultures (data not shown). The fibroblasts subsequently produced substantial quantities of IL-8 in response to LPS treatment (Figure 1). In cells cultured under standard growth conditions and then exposed to LPS for 24 h, approximately twice as much ($P < 0.05$) IL-8 was produced by HR cells in comparison to LR cells. Removal of DNA methylation by

AZA and hyper-acetylation of histone proteins by TSA alone or in conjunction led to an increased responsiveness to LPS evident by substantial increase in the production of IL-8 following LPS treatment (**Figure 1**). The AZA and/or TSA pre-treatments potentiated the LR culture responses more than the responses of HR cultures such that an overall loss of difference between groups was observed across all epigenetic treatments. There were no differences in the number of cells present for the LPS challenge between control and epigenetic modification cultures indicating that the treatment did not affect cell growth or division rates (data not shown).

3.2 Loss of Gene Expression Differences Between LR and HR Groups Following Epigenetic Modification

Real-time PCR was used to compare gene expression of three LR and three HR cultures following exposure to LPS (100 ng/mL) under standard and AZA-TSA treatment conditions. LPS up regulated *IL-8* in both LR and HR groups under standard conditions, however at the 3-hour time point the HR group had higher ($P < 0.05$) expression than LR group (**Figure 2A**). In comparison, cells having undergone epigenetic modification had enhanced *IL-8* expression at the time of LPS addition, with further induction following LPS, but showed no between-group differences ($P = 0.46$) following exposure to LPS (**Figure 2B**). Similarly, an induction of *IL-6* following LPS was observed in all groups, with HR expression of *IL-6* being higher ($P < 0.01$) than LR cultures at 3 hours under control conditions (**Figure 2C**), and yet, following epigenetic modification there were no between-group differences in *IL-6* message at any time point (**Figure 2D**). Finally, *TNF- α* expression was induced by LPS, but was not significantly different between groups at any time point following LPS exposure under control or treatment conditions (**Figures 2E and 2F**).

3.3 Transcriptomic Analysis of Epigenetic Modification

Global gene expression analysis was compared between three LR cultures under standard or epigenetic modification conditions, at 0, 2, 4, and 8 hours following LPS exposure. Only LR cultures were selected due to their greater potentiation of the LPS-induced IL-8 production response following epigenetic treatment as shown in Figure 1. The most sensitive cultures would potentially be more suitable to reveal genes affected by epigenetic modification.

In the absence of AZA-TSA, the cultures responded to LPS with a considerable number of genes altered at each time point following exposure. Within 2 hours of LPS exposure, 241 genes had altered expression (fold change ≥ 1.5 ; FDR < 0.05) in comparison to hour 0 levels. Gene expression measured 4 hours post-LPS indicated 278 genes (**Supplemental File 1**) displaying differential expression in comparison to hour 0; this number dropped to 137 genes by hour 8. Immune associated genes showing a strong response to LPS included *CCL5*, chemokine *CXCL motif (CXCL)-2*, *IL-6*, *IL-8*, and *TNF- α* among others (**Table 2**).

Analysis of the transcriptome was characterized by differential expression (fold change ≥ 1.5 ; FDR < 0.01) of 1406 transcripts (**Supplemental File 2**) due to epigenetic modification prior to LPS treatment (hour 0). Of genes associated with the innate immune response, several were up regulated by more than five-fold due to treatment alone (hour 0) including *CCL-17*, *CXCL2*, *IL-8*, and other genes including *ubiquitin-like modifier ISG15 (ISG15)*,

myxovirus (MX) resistance-1, and *2'-5'-oligoadenylate synthetase (OAS)1* (**Table 2**). In addition, multiple genes involved in spermatogenesis as well as those located on the X chromosome were upregulated following AZA-TSA treatment.

Across all time points 1,758 transcripts were differentially expressed (fold change ≥ 1.5 ; FDR < 0.01) due to epigenetic modification, although, some of these genes showed little difference at hour 0 (262 genes FC < 1.5). This suggests a large regulatory role for methylation and acetylation in innate immune signaling. Within the group of genes showing differential expression between control and AZA-TSA treatments following LPS exposure, several genes associated with TLR4 signaling were up regulated including *IL-6*, *TNF- α* , *CXCL2*, *CCL20*, and *SAA3*, among others. These genes, excluding *CXCL2*, showed no differences in expression between cells grown under standard or AZA-TSA treatment conditions at hour 0. Many genes, such as *TLR4* and *CXCL12* showed no differences in expression due to epigenetic modification.

Functional analysis of genes differentially expressed between control and AZATSA cultures at hours 2, 4, and 8 post-LPS (**Supplemental File 3**) using DAVID identified 12 pathways affected by treatment. These included p53 pathway signaling, fatty acid metabolism, focal adhesion, cytoskeleton regulation, and the cell cycle, among others.

3.4 Validation of microarray results by RT-PCR

Use of real-time PCR confirmed that epigenetic modification of LR cultures with AZA and TSA increased gene expression of *IL-8*, *IL-6*, *TNF- α* , *CCL20*, and *SAA3* ($P < 0.05$) at varying times following exposure to LPS (**Figure 3**). RT-PCR as a more sensitive probe of gene expression showed higher differential expression levels based upon treatment than did microarray analysis. Higher *IL-6* expression due to treatment as determined by microarray increased at hours 0, 2, 4, and 8 from 1.5, 2.5, 2.4, and 1.9 fold respectively to 3.1, 4.8, 4.2, and 4.7 fold. Fold change of *TNF- α* expression due to treatment as determined by microarray was 1.2, 1.7, -1.04 , and -1.1 as compared to 3.2, 13.5, 3.8, and 15.0 fold higher by RT-PCR. A similar increase in differential expression of *CCL20* due to treatment was seen comparing results of microarray analysis (1.2, 3.8, 4.4, and 4.4 fold higher) and RT-PCR analysis (4.0, 16.7, 10.3, and 28.4 fold higher). Differential expression of *IL-8* increased dramatically when analyzed by RT-PCR, especially at the 0 hour time point. While microarray analysis revealed that *IL-8* expression was 6.6, 5.0, 7.3, and 4.3 fold higher at hours 0, 2, 4, and 8 respectively, RTPCR analysis found *IL-8* expression to be 831, 14.0, 25.5, and 13.5 fold higher due to AZA-TSA treatment.

RT-PCR also confirmed that *TLR4* expression was not different ($P = 0.52$) between treatment and control cultures (**Figure 3E**). Analysis also showed that the fibroblast cultures did not increase expression ($P = 0.68$) of *TLR4* following exposure to LPS.

4. DISCUSSION

The ability of an individual to recognize and respond to bacterial components such as LPS during an infection plays a critical role in host defense (Beutler et al., 2003). Previous evidence has indicated that variation in innate immune response capability such as a defects

in the TLR4 signaling pathway may lead to higher disease susceptibility and poorer outcomes (Ku et al., 2007; Medvedev et al., 2003). While our understanding of genetic variation and its role in the innate immune response has been informative, the interface between genetics and environment has been shown to play an important role in this pathway as well (Knight, 2013). A better understanding of how individual variation is regulated across a population could lend to further discoveries of markers playing important roles in disease resistance.

With the advance of sequencing technologies, a large degree of epigenomic variation at the population level has been described across many organisms (Milosavljevic, 2011). The goal of this study was to investigate the role of epigenetic variation on the innate immune response of bovine dermal fibroblast cultures from two phenotypically diverse populations. Establishing a narrowed epigenetic basis for variation in the response to LPS would focus investigations aimed at identifying important biomarkers of the innate immune system. Our study focused on TLR4 signaling by using LPS responsiveness as the investigated phenotype, however it is likely that phenomenon observed here through epigenetic manipulation are more broadly applicable considering common downstream mediators shared between various PAMP and ligand receptors including the family of TLR receptors, IL-1R, and TNF-R (Verstrepen et al., 2008).

The use of epigenetic modifiers has previously been reported to alter cellular response to LPS through a shift in IL-8, TNF- α , and IL-6 production (Chavey et al., 2008; Gillespie et al., 2012; O'Gorman et al., 2010). IL-8 is an important chemoattractant of neutrophils and may be produced by a wide array of cell types following an infection (Andersen, 2007; Herndon et al., 2010; Lahouassa et al., 2007). IL-6 and TNF- α play roles in inflammation and the strength of production following infection has been linked closely to pathogen clearance (Zanotti et al., 2002). The importance of these and other cytokines in the response to infections highlight the importance of understanding how variability in their production contributes to disease progression. The present study focuses on how alteration of epigenetic markers may affect between-animal variation in the immune response. The presence of AZA and TSA dramatically increased cellular responsiveness to LPS, without altering TLR4 expression, indicating an inhibitory role of DNA methylation and acetylation acting on the TLR4 signaling pathway. In addition, this increase in LPS responsiveness was greater in LR than HR fibroblasts. Under AZA, TSA, or AZA-TSA conditions, the differential response to LPS between LR and HR cultures observed under standard conditions was abolished suggesting between-group differences in DNA methylation and acetylation as causative for the different IL-8 response phenotypes observed under standard conditions. The differential change between the extreme phenotypes appears to indicate that a high degree of variation exists in epigenetic modulators of gene expression across populations. Previous work has focused on epigenetic variation comparing healthy individuals to those affected by a specific disease (Andia et al., 2010; Gillespie et al., 2012; Ishida et al., 2011); our data aims to identify variability in the general population of cows that could affect susceptibility to a large number of pathogens. While IL-8 protein and mRNA variation between the pre-selected groups was expected, we also identified differential expression of *IL-6* between LR and HR cultures following LPS exposure for 3 hours. *TNF- α* expression was not different (P

= 0.08) between groups likely due to greater variability between individuals within each group. We and others have shown that the timing of TNF- α induction following LPS is much more acute and short-lived than other LPS-responsive genes (Carroll et al., 2009; Lee et al., 2006). Following treatment with AZA and TSA, the between-group differential expression of *IL-8* and *IL-6* was lost. The overall effect of AZA and TSA on the response of both LR and HR fibroblasts to LPS suggests that cultures from either group are heavily regulated by both methylation and acetylation. Both LR and HR cultures, in response to epigenetic modification, displayed significantly higher responses to LPS indicating that it is likely differing degrees of epigenetic regulation between groups rather than a total absence in HR cultures that leads to the different phenotypes seen.

Microarray analysis of gene expression comparing control and AZA-TSA cultures identified over 1,700 genes that were differentially expressed due to the epigenetic modifications. It is important to note however, many of these genes (845 genes) were upregulated (Fold Change 1.5; FDR < 0.01) or repressed (561 genes) by AZA-TSA alone prior to LPS exposure. For the upregulated genes this likely reflects the loss of transcriptional repression due to DNA methylation and histone acetylation. When accounting for the interaction of AZA-TSA with LPS exposure, 828 genes were significantly (FDR < 0.01) altered. This large subset of LPS responsive genes modified by AZA and TSA suggests that epigenetic markers heavily regulate a large number of them. Genes responsive to our treatments typically fell into two general profiles. The first included genes that were up regulated by AZA-TSA before the addition of LPS into culture. In this group, expression increased without any other external stimulus being required indicating that under control conditions their expression was repressed by epigenetic markers. Immune associated genes such as *IL-8*, *CXCL2*, and *MX1* fell into this category. This correlates with previous data showing a similar increase in *IL-8* expression levels due to methylation and acetylation modifications alone, prior to any other stimulus (Angrisano et al., 2010). *IL-8* protein was undetectable in AZA-TSA cultures following 24-hour incubation in control media (lacking LPS) suggesting that while transcript levels had risen, protein levels were still below the ELISA limit of detection. This may be due to an increase in transcript levels due to AZA-TSA exposure without the requisite transcript stabilization associated with a response to LPS controlled by the p38 mitogen-activated protein kinase (MAPK) pathway (Hoffmann et al., 2002; Li et al., 2002). This would account for the high transcript levels seen prior to the addition of LPS, but a lack of protein due to an unstable transcript before to translation. A second general response includes genes upregulated due to epigenetic modification, but only following exposure to LPS. These genes were seemingly unaffected by AZA-TSA treatment alone, and required a secondary stimulus (LPS) for differences to be seen. This profile would seem to indicate that our treatment removed transcription inhibitors, but did not lead to gene induction. A striking example of this is seen with *SAA3*, which was largely unresponsive following LPS exposure in standard conditions but was markedly up regulated by LPS following AZA-TSA treatment. This would suggest that without AZATSA, up-regulation of *SAA3* transcription is delayed due to requisite removal of epigenetic regulators blocking signaling. The typical *SAA3* response profile following innate immune stimulation is delayed by several hours compared to more acute responses of inflammatory cytokines (Larson et al., 2005).

Although an appreciable number of genes were affected by the epigenetic treatment in the response to LPS, it is interesting to note that many of them are downstream response proteins such as cytokines and chemokines while genes within the TLR4 signaling pathway were largely unaltered. Previous work investigating differential gene expression of high and low responsive dermal fibroblast cultures showed no differences in TLR4 signaling, but significant variation between groups in downstream response genes (Kandasamy and Kerr, 2012). In addition to genes involved in the innate immune response, functional analysis showed genes altered by AZA-TSA exposure following LPS exposure were associated with a number of pathways including cell adhesion, cell cycle, and fatty acid metabolism. This would suggest that AZA and TSA are having broad effects on our culture system to a greater extent than simply the immune response and TLR4 signaling.

General mechanisms to account for differential responsiveness between LR and HR groups include one of two potential mechanisms. One would be simply through a differential regulation in the transcription of LPS responsive cytokines such as *IL-6*, *IL-8* and *TNF- α* . Another may involve an indirect mechanism such as differential kinase activity, which is key to phosphorylation and activation of the mediators found in the response prior to NF κ B activation such as MyD88 or interleukin-1 receptor associated kinase (IRAK-1). This would not require differential expression of these mediators, but would still lead to differences in activation of NF κ B responsive genes.

Among the genes affected by AZA-TSA treatment before the addition of LPS, a number control for X chromosome inactivation and spermatogenesis. It is interesting to note as all samples were from females, and may be used to highlight the importance of epigenetic silencing in sex differentiation. In females, a single X chromosome is silenced to ensure that X gene expression is not doubled in comparison to males (Kelkar and Deobagkar, 2010). Several X antigen family genes, including member 5, were up regulated as well as *chromosome X open reading frames 23, 26, and 57*. The gene *X-inactive specific transcript (Xist)* has been implicated as a master control for X chromosome inactivation (Jeon et al., 2012), and while upregulated due to AZA-TSA in our model, it appears as though DNMT and HDAC inhibition played a larger role in the reactivation of the silenced X genes. Prior research has hypothesized that errors in the epigenetic silencing of the X chromosome may cause autoimmune diseases and rheumatoid arthritis (Chabchoub et al., 2009). While this may be the case, it is difficult to form a strong connection between the increase in X inactivated genes and the heightened response to LPS in our model. In addition to X inactive genes, several involved in spermatogenesis including *deleted in azoospermia-like (DAZL)*, *spermatogenesis associated 22 (SPATA22)*, spermatogenesis and oogenesis specific basic helix-loop-helix 2 (*SOHLH2*), and *sperm acrosome associated 1 (SPACA1)* were highly upregulated due to AZA-TSA treatment. Again, while important to note as sex determinant genes heavily regulated by epigenetic silencing, it is unlikely that they played a major role in the alteration of the innate immune response within our data set.

While important markers of the immune response were investigated in relation to epigenetics, this study did not attempt to define specific areas of variation leading to the phenotypic differences described. AZA and TSA are global modulators of epigenetic markers and while effective in reducing variation, their use makes it difficult to pinpoint a

specific area important in this effect. Future studies should focus on where differences in DNA methylation or histone acetylation occur between low and high responsive cultures to better define potential mediators of this variable response to LPS.

5. Conclusion

This study is an important first step towards the understanding of epigenetics and individual variation in the innate immune response via TLR4 signaling. The loss of variation between low and high LPS responsive cultures in IL-8 production and *IL-6* transcription due to epigenetic reprogramming suggests that a majority of phenotypic variation is regulated by this mechanism. In addition the description of genes within our model modified by AZA and TSA treatment in the response to LPS helps to define areas of the innate immune response regulated by epigenetic markers. Increasing our understanding of the epigenetic regulation of LPS response will make the identification of potential environmental stimuli affecting disease susceptibility more realistic. In doing so, this knowledge may be an important key to reducing disease susceptibility in both production animals as well as humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fibroblast response to LPS under control conditions or following epigenetic modification

Production of IL-8 by dermal fibroblasts following exposure to LPS (100 ng/ml) for 24 hours after undergoing pre-treatment with media alone, AZA, TSA, or AZA-TSA. Animals displaying differential responsiveness to LPS (Low responders and High responders; $n=3/\text{group}$) were investigated. In the absence of AZA and TSA, low and high responders showed differential responsiveness to LPS. However, following exposure to AZA and TSA either alone or in conjunction, differences in responsiveness to LPS were lost between the groups. In addition, exposure to AZA and TSA alone or in conjunction significantly increased IL-8 production in response to LPS. IL-8 production in the absence of LPS was not detectable. * indicated $P<0.05$. All values are mean $\pm$ S.D. ($n=3/\text{group}$).

Figure 2. Fibroblast response to LPS as measured by RT-PCR under control conditions or epigenetic modification

Comparison of gene expression following exposure to LPS at hour 0 for low and high responding cultures under both control (left panel) and epigenetic modification (right panel) conditions. IL-8 (A and B), IL-6 (C and D), and TNF- α (E and F) mRNA were all targeted. Values are expressed as dCt using β -actin expression as the endogenous control. All values are mean $\pm$ S.D. (n=3/group). * indicates significantly different expression levels ($P < 0.05$).

Figure 3. Confirmation of microarray by RT-PCR

Expression of IL-8 (A), IL-6 (B), TNF- α (C), CCL20 (D), TLR4 (E) and SAA3 (F) mRNA following exposure to LPS for three low responding cultures. Expression was analyzed for response under both control and epigenetic treatment conditions. * indicates significantly different ($P < 0.05$) gene expression due to epigenetic modification. Values are expressed as dCt using β -actin expression as the endogenous control. All values are mean $\pm$ S.D.

Table 1

Primer pairs used for amplification of target genes by RT-PCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Reference
IL-8	GCTGGCTGTTGCTCTCTTG	AGGTGTGGAATGTGTTTTATGC	(Pareek et al., 2005)
IL-6	TGAGGGAAATCAGGAAAATGT	CAGTGTGTTGTGGCTGGAGTG	(Pareek et al., 2005)
TNF	TCTTCTCAAGCCTCAAGTAACAAGC	CCATGAGGGCATTGGCATACTAC	(Bougarn et al., 2011)
SAA3	CCTCAAGGAAGCTGGTCAAG	TACCTGGTCCCTGGTCATACTAC	(Bougarn et al., 2011)
CCL20	TTCGACTGCTGTCTCCGATA	GCACAACCTGTTTCACCCACT	(Bougarn et al., 2011)
TLR4	ACTGCAGCTTCAACCGTATC	TAAAGGCTCTGCACACATCA	(Ibeagha-Awemu et al., 2008)
B-Actin	GCAAAATGCTTCTAGGCGGACT	CAATCTCATCTCGTTTTCTGCG	(Pareek et al., 2005)

Table 2

Expression of selected genes either responsive to LPS or exhibiting differential expression due to AZA-TSA treatment.

		Control Conditions (vs. Hour 0)			AZA-TSA (vs. Hour 0)		
Gene Symbol	AZA-TSA vs Control (Hour 0)	Hour 2	Hour 4	Hour 8	Hour 2	Hour 4	Hour 8
Chemokines							
CCL2	3.32 **	27.49 **	20.36 **	18.99 **	16.80 **	13.76 **	9.93 **
CCL5	1.22	1.48	1.81 *	1.85 *	2.55 **@	2.37 **	2.50 **
CCL17	8.32 **	1.02	1.00	−1.05	1.03 @	1.08 @	1.08 @
CCL20	1.22	1.42	1.53	1.27	4 43 **@	5.60 **@	4.56 **@
CXCL1	10.41 **	100.08 **	44.65 **	40.86 **	11.81 **	8.36 **	8.17 **
CXCL2	25.82 **	89.88 **	69.92 **	58.35 **	6 24 **@	6 70 **@	5 94 **@
CXCL3	4.28 *	40.85 **	12.24 **	10.27 **	15.68 **	7 48 **@	5.85 **
CXCL5	4.09 *	4.47 **	8.87 **	10.60 **	2.19 **	3.55 **@	4.81 **
IL8	6.58 **	10.17 **	4.74 **	9.17 **	7.78 **@	5.25 **@	5.98 **@
Cytokines							
IL1A	1.31	4.31 **	2.61	1.94	7.16 **	3.94 **	2.30
IL1F6	1.22	1.41	2.11	3.44 **	1.54	2.80 *	2.53 *
IL-1RN	3.16 **	1.28	1.24	−1.06	1.32 @	1.39 @	−1.06 @
IL6	1.46	6.81 **	4.92 **	5.08 **	11.63 **@	8 19 **@	6.66 **
TNF	−1.25	1.93 *	1.55	1.28	2.74 **	1.26	−1.13
Anti-viral							
IFIH1	8.06 **	1.04	2.40 *	3.29 **	1.36 @	1.38 @	1.66 **@
ISG15	24.82 **	1.85	2.36 *	3.58 **	1.14 @	1.20 @	1.30 @
OAS1	47.76 **	1.85	2.67 *	7.61 **	1.29 @	1.26 @	1.65 @
MX1	25.30 **	1.10	1.49	2.76 *	1.12 @	1.15 @	1.27 @
TLR Response Pathway							
CD40	1.18	2.16 *	2.60 *	2.45 *	1.90	1.98 *	2.02 *
NFKB2	−1.22	1.52 *	1.46 *	1.45 *	2.15 **	2.31 **	2.03 **
NFKBIA	−1.04	7.66 **	3.94 **	3.32 **	9.49 **	5.93 **	3.41 **
NFKBIZ	1.05	5.77 **	2.28 *	2.18 *	10.36 **	4.66 **	2.98 **
Acute Phase Response							
SAA3	−1.06	1.15	2.06	3.13 *	13.78 **@	23.98 **@	22.42 **@

		Control Conditions (vs. Hour 0)			AZA-TSA (vs. Hour 0)		
Gene Symbol	AZA-TSA vs Control (Hour 0)	Hour 2	Hour 4	Hour 8	Hour 2	Hour 4	Hour 8
Down-regulated by AZA-TSA							
BRCA1	-1.99 *	-1.34	-1.24	1.09	-1.19	-1.55	1.13
IRAK4	-1.43 *	-1.31	-1.07	1.05	-1.13	1.03	1.24 *
PTX3	-1.14	1.69 *	2.26 **	2.34 **	1.74 *	2.09 **	1.87 *

Data obtained by microarray analysis and represented as fold change comparing expression between control and AZA-TSA cultures at hour 0 or as fold change comparing hours 2, 4, and 8 within each treatment to expression at hour 0. A positive fold change indicates either higher expression in AZA-TSA cultures (0 hour comparison) or a higher expression than hour 0 (LPS response)

** FDR < 0.01

* FDR<0.1

@ indicates differential expression between AZA-TSA and control culture at specified time point FDR<0.01.