

Cellular Immuno-Genomics: A Novel Approach to Examine Genetic Regulation of Disease Resistance in Cattle

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Summary:

The immune system is a complex network of cells and molecules protecting the host against pathogens in a delicately coordinated process. Diversity in response and different layers of defence mechanisms ensure survival of the host in the face of diverse pathogen challenge. This system is controlled by more than 6000 genes, making the immune system complex in terms of understanding the nuances of its genetic regulation. Resistance to disease is even more complicated as pathogens have their own set of genes that interact with the immune system in any given environment. Advances in genomic methods have opened a new opportunity to study genetic regulation at the cellular level. Utilizing this concept to study the cells of the immune system in the face of pathogenic challenge is a useful approach to examine the genetics of disease resistance. In the current study, two bactericidal responses of bovine monocyte-derived macrophages (MDMs), nitric oxide production (NO-) and phagocytosis, against *Escherichia coli*, were investigated. The results demonstrated notable individual variation for both responses (coefficient of variation 35% in phagocytosis ability and 65% in production of NO-); as well as a strong, significant, and positive correlation between NO- production and phagocytosis (Spearman's rank correlation: p value = 0.01, $\rho = 0.62$). At the next step, MDMs derived from 8 Holstein cows were divided into high, average, and low responder phenotypes, based on their production of NO-. The MDM transcriptome of these three phenotypes were compared to the untreated controls at 3 hours after *E. coli* challenge. More than 1300 genes were identified to be differentially expressed after the challenge (FDR p-value ≤ 0.05). The gene ontology analysis revealed different underlying molecular mechanism that are associated with impaired or enhanced ability to elicit bactericidal response. Of particular interest was the anti-inflammatory pathway which includes the production of IL10 that was enriched in the low and average responder phenotypes, while genes in the Tumour Necrosis Factor - α pathway, a master regulator of pro-inflammatory cytokine production, were enriched in MDMs of the high responder phenotype. The findings from this study showed the possibility of using a cellular immuno-genomic approach to examine immune functions in cattle. The next step of this study will be investigating the genetic polymorphisms within bovine MDMs that are causing or associated with the gene expressions and pathways that were found to be associated with the high, average or low phenotypes based on NO- production.

Keywords: Macrophage, Disease Resistance, RNA-Seq, Cattle, Nitric oxide production, phagocytosis

Introduction

Despite the achievements to improve production traits in livestock, health traits have remained relatively unchanged. Infectious diseases are still a major source of economic

losses in livestock, as well as a major concern for animal wellbeing (Chesnais et al. 2016). Recently in Canada, direct measures of resistance to mastitis, the most prevalent infectious disease in dairy cows, has been included in the selection program. However, the relatively low heritability and its low weight in the selection index are not promising to initiate a notable improvement in the near future (Mallard et al. 2015). The complexity of host-pathogen-environment interactions is a major limiting factor in identifying markers with strong association with resistance to infectious diseases. Each element of this interaction can introduce prominent errors into the case-control studies, weakening the associations and lowering the heritability estimates. For instance, infectious dose, pathogen load, time of infection, mutation of pathogen during the course of infection, and immunological memory are the key limiting factors in detecting reliable markers (Bishop and Woolliams 2014). Although experimentally infecting animals with a consistent pathogen dose in a controlled environment can overcome some of these limitations, it is still not the same as natural infection, and challenge experiments are costly, as well the ethical concerns limit the feasibility of these experiments, especially for large animals.

A method that can provide additional information on the genes and pathways involved in host defence is through the use of cellular immuno-genomics. In this approach, genomic control of the immune system at the cellular level is investigated in an *in-vitro* or *ex-vivo* setting. In an *in-vitro* setting, one cell type is purified from the host, and is challenged with pathogens of interest to identify genetic markers that control the performance of that cell (Zeller et al. 2010; Banos et al. 2013; Ko and Jaslow 2014). However, such experiments need critical consideration of the role of the cell in the grand scheme of the immune response, rigorous optimization of the *in-vitro* condition to mimic the *in-vivo* condition, and meticulous interpretation of the results.

In the current study, it was hypothesized that variation within *in-vitro* responses of monocyte-derived macrophages (MDMs) against *Escherichia coli* (*E. coli*) is genetically controlled by utilizing distinct intra-cellular molecular pathways. To test this hypothesis, an all-synthetic culture system was developed to grow bovine MDMs. Then, MDMs were exposed to *E. coli* and the bactericidal responses were measured. As a second step, the transcriptome of MDMs were analyzed to identify underlying molecular mechanisms and the key genes involved.

Material and Methods

Experiment I - Phenotypic Characteristics of MDMs

Generation of MDMs

Blood samples were collected from the tail vein of 16 Holstein mid-lactating cows from the research herd at the University of Guelph. Blood Mononuclear Cells (BMCs) were purified based on the gradient centrifuge separation method and cultured at for 2 hours in Monocyte Attachment Medium at 37 °C. Non-adherent cells were removed by washing and the medium was replaced with AIM V® Medium in the presence of 5ng/ml recombinant bovine GM-CSF and 5% CO₂. After 6 days of incubation, adherent cells were detached from the flask using TrypLE™ Select Enzyme. Characteristics of MDM (strong Auto-fluorescence, CD14⁺, CD205⁻) were analyzed using flow cytometry to determine the proportion of MDM among the harvested cells.

Analyzing the Performance MDMs

1 – Phagocytosis

MDMs from each cow were seeded in nine wells of an opaque 96-well plate in AIM V® Medium. These nine wells were assigned into one challenge group and two control groups, each group including three replicates. The challenge group was exposed to pHrodo™ Green conjugated *E. coli* (K-12 strain, MOI:5) for four hours. One of the control groups was labeled with NucBlue™ Live ReadyProbes™ Reagent as an indicator of the number of MDMs and the other control group was assigned as a cell-only negative control to correct for auto-fluorescence. The plates were washed 3 times before reading and then the fluorescence intensity (FI) was measured by a microplate reader. The average FI of each sample was corrected by the cell-only control group and normalized against the FI of the control group containing the NucBlue™.

2 -Nitric Oxide Production

MDMs from each cow were seeded in 2 wells of a 48-well plate in AIM V®. One well was assigned as the negative control (cell-only) and the other well was exposed to *E. coli* (an isolate from a cow with clinical mastitis in Canada, MOI: 5) for 48 hours. Supernatant from each well was collected and the concentration of nitric oxide (NO-) was in 3 technical replicates measured with the Measure-iT™ High-Sensitivity Nitrite Assay Kit. The concentration of NO- in the challenge group was corrected by the control group. In addition, the accuracy of the results was tested by repeating the procedure in its entirety on new samples from the same cows (n = 4).

Statistical analyses

The correlation between the phagocytosis and NO- production in response to *E. coli* was investigated using Spearman's rank-order correlation and Pearson correlation coefficients. These coefficients and their p-values were calculated in SAS (SAS Inc, V 9.4) by employing the PROC CORR procedure. In addition, PROC GLM was utilized to determine the amount of variation in NO- production that is explained by the phagocytosis (uptake of the bacterium) using the following model:

$$y_{jk} = \mu + g_j + \beta_1 \times p_k + \beta_2 \times a_k + e_{jk}$$

Where y_{ijk} is the vector of response of the k^{th} animal; g_j is the fixed effect of j^{th} sampling group; β_1 is the linear coefficient of the fixed regression on phagocytosis (p_k) of the k^{th} animal; β_2 is the linear coefficient of the fixed regression on age (a_k) of the k^{th} animal (in months); e_{ijk} is the random residual effect.

Experiment II - Transcriptomic Analysis of MDMs

Phenotypic Classification of MDM

Nitric oxide concentration was chosen as the indicator of bactericidal performance of MDMs in response to *E. coli*. This indicator showed the highest variation as well as strong correlation with phagocytosis. Based on the results, 8 samples were selected from the first experiment to represent three phenotypes - High (H), Average (A) and Low (L) with mean NO- concentration of 13.1, 6.3, and 4.4 mM, respectively (Figure 1).

RNA Purification and cDNA Library Preparation and Sequencing

MDMs were exposed to *E. coli*, as described in the previous section, and one untreated

control group was included. At 3 hours after challenge, the total RNA was extracted using TRIzol according to the manufacturer's protocol. The quantities of the purified RNA samples were measured by the RNA High sensitivity kit in the Qubit system and the qualities were checked by the Agilent 2100 Bioanalyzer. cDNA libraries were prepared using TruSeq stranded mRNA Libraries Prep kit (Illumina) and each sample was labeled with a unique index. Equal amount from each of the 16 libraries were pooled together and were paired-end sequenced in one lane of Illumina HiSeq 4000 system.

Bioinformatics and Functional Annotation of Differentially Expressed genes

The sequencing results were analyzed using CLC Bio software (Qiagen). The reads were first de-multiplexed and filtered for quality. Then, the reads that passed the QC were mapped to the bovine UMD 3.1 reference genome (release 89 from Ensembl). The heat map from challenged samples was generated along with the similarity tree based on the expression of all genes. Differential expression in fold change (FC) for each gene was determined against the control group within each phenotypic group. Genes with False Discovery Rate (FDR) corrected p-value of less than or equal to 0.05 for all three groups were selected for further investigation. At the next step, the differences between FCs were compared among the phenotypic groups. Genes with absolute FC difference of less than 1.5 between all three groups were clustered together as the common genes. Genes with absolute FC difference of greater than 2 between each phenotype were clustered as the phenotype-specific genes for each phenotype. Each cluster of genes was analyzed for gene and functional annotations using the innatedB Gene Annotation service.

Results

I. Phenotypic Characteristics of MDMs

Tissue resident macrophages play a crucial role in controlling pathogens as the first line of defence. They are also professional antigen presenting and as such are important in initiating adaptive immune responses. Macrophages can destroy the pathogens via phagocytosis and secretion of microbicidal molecules. In this experiment, more than 95% of the harvested cells represented the characteristics of macrophages, indicating uniformity of the cell population. Then, phagocytosis and the production of NO⁻ were investigated when MDMs were exposed to *E. coli*. The statistical analysis did not show any effect of age or sampling group on the phagocytosis, or on the production of NO⁻. In addition, the mean deviation of NO⁻ concentration from repeated samples (technical variation) was less than 0.5mM. In contrast, considerable variation was observed among individuals in both phagocytosis and NO⁻ production. The coefficient of variation (CV) ranged from 35% in phagocytosis ability to 65% in production of NO⁻. The Pearson correlation between phagocytosis and the concentration of NO⁻ in the culture supernatant was 0.60. However, the R-square of the statistical model was 0.99 when all the fix effects were included. In addition, the rank correlation between these two traits was statistically significant (p value = 0.01), positive, and strong ($\rho = 0.62$).

II. Transcriptomic and Functional Analysis of MDMs

Messenger RNA (mRNA) is the intermediating molecule between the genomic information and proteins functioning within the cell. The expression of mRNA molecules is dynamic and represents genes and pathways that cells utilize to generate a response. In this study, the

transcriptome of MDMs were analyzed 3 hours after being challenged with *E. coli* in order to study the early responses of the MDMs to this pathogen. The basic bioinformatics results of the sequencing are summarized in the table 1. The similarity tree based and the heat map from challenged samples showed the phenotypic groups are falling into 3 distinct branches (Figure 2). From more than 14,000 genes that were expressed in the *E. coli* exposed samples, 916 genes were differentially expressed compared to the unchallenged samples ($FDR \leq 0.05$ in all three groups). Among these genes, 417 genes were clustered in the common genes group, 241 genes in the H, 156 genes in the A, and 140 genes in L (Figure 3). The gene ontology (GO) analysis showed that the most over represented terms among the common genes for the cellular compartment, molecular function, and biological process were “nucleus”, “protein binding” and “positive regulation of I-kappaB kinase/NF-kappaB signaling”, respectively. The GO analysis on the phenotype-specific clusters (H, A, and L) resulted in similar over represented terms for the cellular compartment and molecular function, “extracellular space” and “cytokine activity”, respectively. The most over represented GO term for the biological process for the H phenotype-specific cluster was “cellular response to lipopolysaccharide” but “immune response” for A and L phenotype-specific clusters. These results showed that the first difference among the phenotypes is related to the biological processes rather than molecular functions or a specific compartment of the cell. Further pathway analysis to investigate the role of these genes in biological pathways revealed a notable difference among these phenotypes. The most over represented pathway for the cluster of common genes was the “TLR4 cascade”. TLR4 cascade is the main cellular pathway in recognition and initiation of response to lipopolysaccharide, the most abundant molecules on the surface of *E. coli*. The pathway of Tumor Necrosis Factor - α (TNF- α) was only present in the H phenotype-specific cluster. While, “IL23-mediated signalling events”, and “IL27-mediated signalling events” were more represented at in the A and L phenotype-specific clusters.

Discussion

The primary role of the immune system is to protect the host against pathogens and cancer. This system is comprised of complex networks of cells and molecules that detect and respond to danger. The performance of this system along with its interactions with pathogens and environment during the course of infection determines susceptibility or resistance to diseases (Mallard et al. 2015; Stear et al. 2017). However, the overall performance of the immune system is not easily measurable due to its complexity. The immune responses are initiated in the infected tissue and expansion of the response continues in the secondary lymphoid tissues. The effector molecules, such as antibodies and cells, including activated lymphocytes, then can use blood vessels to circulate back to the infected sites (Randolph et al. 2005). Measuring antibody in the blood is a good indicator of antibody-mediated immune response since these are the effector molecules secreted by B-lymphocytes. However, analyzing the circulating cells that are responsible for cell-mediated adaptive immune responses are not that reliable an indicator of cellular immunity since the effector molecules of these cells may not be secreted and tend not to be active when the cells are passing through the blood (Thompson-Crispi et al. 2012). Measuring the cell-mediated innate immune responses are even more complicated due to the diversity of cell types and that the phenotype of some cells change when they migrate into the tissues; for example, monocytes may transform into tissue macrophages (Italiani and Boraschi 2017). Therefore, measuring the performance of the cells of the innate immune system is more feasible using an in-vitro experiments. However, designing a system that mimics the in-vivo condition and still

represents the individual genetic variation is challenging. As an example, MDMs have been the subject of many studies, but almost all culture systems contained fetal bovine serum (FBS). Although FBS helps the cells to grow *in-vitro*, there are some components that alter the function of these cultured cells, such as exosomes and transforming growth factors (Oida and Weiner 2010; Beninson and Fleshner 2015). In some cases, the cultured MDMs receive two opposite signals when they are cultured in the presence of the colony stimulating factor 1 (CSF-1) and then exposed to a pathogen (Thirunavukkarasu et al. 2015). To overcome this limitation, in the current study a serum-free all-synthetic culture system was optimized to culture monocytes and transform them to MDMs more representative of *in-vivo* conditions. The notable variation that was observed for the both microbicidal indicators, phagocytosis and NO⁻ production, showed that this system is not overriding the genetic control of the MDM performance. Considering the strong functional association between these indicators with neutralization of the pathogens at the early stage of the infection, this system has the potential to more accurately identify the bovine genes that are associated with resistance to diseases, as it has been shown in other species (Sun et al. 2008; Ma et al. 2010; Akhtar et al. 2016). In addition, innate defences are initiated by recognizing the pathogen's associate molecular patterns (PAMPs) that are often in common between groups of pathogens. Therefore, the results from this study, which is the first to utilize the cellular immuno-genomic approach in livestock, are likely more representative of resistance to a broad range of pathogens with PAMPs common to *E. coli*.

The second part of this study was to perform transcriptomic analysis of the MDMs cultured in serum-free conditions. The results of this experiment confirmed the reliability of this system to identify genes and pathways that control important macrophage responses. The similarity tree that was generated using the expression of all genes was reliably matched to the phenotypic groups. In addition, the pathway enrichment analysis showed notable differences in the molecular pathway in the three different NO⁻ phenotypic groups. The pathway of anti-inflammatory cytokines production (such as Interleukin 10 and 27 (IL10 and IL27, respectively) were enriched only in the L and A phenotype-specific clusters that also produced the least amount NO⁻. In contrast, the pathways that lead to the expression of genes with positive effects on inflammation (TNF- α) were observed in the MDMs with higher production of NO⁻ (i.e. the H phenotype). In addition, genes with negative impact on apoptosis (cell death) were only present in the H phenotype, showing this phenotype is more resistance against the lipopolysaccharide induced apoptosis. A transcriptomic study on LPS stimulated sheep bone marrow-derived macrophages (BMDM) found enrichment of "response to polysaccharide", "Toll-like receptor signalling", and "IL27" pathways are associated with the response of BMDM to LPS (Clark et al. 2017). The "response to polysaccharide" pathway was identified in the current study in the H phenotype-specific cluster, and IL27 was identified in the A and L phenotype-specific clusters. The "Toll-like receptor signalling pathway" was enriched in all phenotype-specific clusters which shows the crucial role of this pathway in response to LPS/*E. coli*. However, the current study identified that expression of 3 members of this pathway, phosphoinositide-3-kinase regulatory subunit 5, nuclear factor kappa B subunit 1, and CD40 molecule are 3 to 5 folds higher in the H phenotype compared to A and L phenotype. This difference shows the ability of the cellular immuno-genomic approach to identify the pathways that are associated with the cellular phenotype of macrophages.

In conclusion, the results of this study align with the hypothesis that individual variation in bovine MDM function (phagocytosis and NO⁻ production) is detectable using a serum-free *in-vitro* culture system and that this variation is associated with the different molecular pathways detected using transcriptomic analysis in MDMs classified as H, A or L based on production of NO⁻. The next step is to identify the genes or genetic markers that are

associated with these cellular pathways.

Table and Figures

Table 1- Basic bioinformatic results using of mRNA-seq (n = 16 libraries from 8 cows) to identify molecular pathways associated with bovine Monocyte-derived Macrophage (MDM) functions (phagocytosis and NO-).

Read length (bp)	Average number of reads per library	Average mapped reads to a unique gene per library	Average number of genes with RPKM > 0.2		Number of differentially expressed genes (FDR p-value ≤0.05)		
			Control	Challenge	High	Average	Low
150	53.930e6	65%	11,718	11,573	1,315	1,817	1,387

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