Expression of TLR2 Pattern Recognition Receptor on Mononuclear Cells of Dairy Cattle Ranked Using Estimated Breeding Values (EBV) of Adaptive Immune Response Traits

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ABSTRACT: Toll-like receptor 2 (TLR2) is a pattern recognition receptor expressed on the surface of epithelial cells and some leukocytes. Binding of bacterial ligands such as lipoproteins and lipopeptides to TLR2 induces proinflammatory innate responses and contributes to the development of adaptive immune responses. In the present study we investigate whether expression of TLR2 by blood mononuclear cells differs among Holstein dairy cows classified as high (H), average (A), and low (L) immune responders, based on their EBVs for antibody-mediated (AMIR) and cell-mediated (CMIR) immune responses. Cells were stained using fluorochrome-labeled monoclonal antibodies, and characterized using flow cytometry. A significantly higher proportion of unstimulated mononuclear cells from H-AMIR cows expressed TLR2, compared to cells from L-AMIR, and H-CMIR cows. Expression of TLR2 may contribute to the enhanced antibody responses and reduced incidence of clinical disease reported for H-AMIR cows.

Keywords: dairy cattle; TLR2; Innate

Introduction

Optimal host defense begins with the innate system and includes barriers such as the epithelium as well as enzymes, proteins and cells that have the ability to respond to a first encounter with a pathogen. Effective signalling by the innate system leads to the influx of intermediary cells such as neutrophils and macrophages, leading eventually to the development of an effective adaptive immune response. The adaptive immune system has the hallmarks of memory, specificity, diversity and self-nonsel recognition. The innate and adaptive systems are interconnected and allow the host to respond to an invading pathogen with some general reactivity on first exposure, but with exquisite specificity on second and subsequent exposures.

The host is alerted to the threat of an invading pathogen through the activation of pattern recognition receptors (PRRs). Both vertebrates and invertebrates express PRRs. They can recognize the molecular patterns that are conserved among pathogens (Takeda et al. (2004)). Toll-like receptors (TLR) are a group of PRRs expressed by epithelial cells and leukocytes, that bind to pathogen associated molecular patterns (PAMPs). When these receptors bind to PAMPs a series of events occurs that transduces a signal to the nucleus, resulting in expression of cytokines including type I interferons and chemokines (Kumar et al. (2011)). These cytokines attract other innate cells such as neutrophils, macrophages and dendritic cells that also express TLRs on their cell surface. Macrophages and dendritic cells, under the influence of TLR signaling and cytokines, act as antigen presenting cells, activating T helper cell sub-populations. T helper 1 (Th1) cells predominately produce cytokines that generate a cell-mediated immune response (CMIR) to respond to intracellular pathogens such as Mycobacterium avium ssp. paratuberculosis (the cause of Johne’s disease). In contrast, T helper 2 (Th2) cells produce cytokines that tend to generate an antibody-mediated immune response (AMIR) towards extracellular pathogens such as Escherichia coli known to cause mastitis.

Work by Wagter et al. (2000) has shown that cows with a high AMIR have less disease, and a recent study of 58 dairy herds (part of the Canadian Bovine Mastitis Research Network) has shown that high AMIR responders have a lower incidence of mastitis compared to average and low responders and tend to have less severe mastitis (Thompson-Crispi et al. (2013)). A study in a large dairy in the USA, has also documented a lower incidence of mastitis, ketosis, metritis and retained placenta in cows with high AMIR and CMIR in comparison to average or low responders (Thompson-Crispi et al. (2012)). Therefore the objective of this paper was to evaluate TLR2 expression in the context of High (H), Average (A) and Low (L) immune response for both AMIR and CMIR immune response traits, and to compare TLR2 expression in blood leukocyte subsets between H-AMIR and H-CMIR groups.

Material & Methods

Animals. Twenty eight cows previously phenotyped for AMIR and CMIR were classified by their estimated breeding value (EBV) into High, Average, and Low groups based on a procedure described by Thompson-Crispi et al. (2013). Cows with an EBV of above +1 or below −1 standard deviation from the mean were considered high immune responders or low immune responders respectively, and thus comprised the following groups: H-AMIR/A-AMIR/L-AMIR and H-CMIR/A-CMIR/L-CMIR.

Cell Isolation and Staining. Mononuclear cells were isolated from whole blood using SepMate tubes and Histopaque 1077 density gradient solution. Cells were counted and re-suspended in PBS+0.5% BSA. Cells were then added to 96 well round bottom plates at 1.0x10⁵ cells per well, for a total of 9 wells per row. The first well was
an unstained negative control, the second an isotype control to detect non-specific binding of mouse IgG1 (AbDSerotec). The next four wells were single stained in this order: human anti-bovine TLR2 (CD282) conjugated with FITC (AbDSerotec) monoclonal antibody (mAb), then mouse IgG1 anti-bovine CD14 (monocytes; VMRD) mAb, mouse IgG1 anti-bovine BAQ155A (a pan B cell marker; VMRD; (Galeotti et al. (1993)) mAb; and mouse IgG1 anti-bovine CD3 (a pan T cell marker; VMRD) mAb. The final three wells were double stained for TLR2 in combination with CD14, BAQ155A, and CD3. The secondary antibody used to label the mouse mAbs was goat anti-mouse IgG1 conjugated to Phycoerythrin-Texas Red (Invitrogen). Cells were incubated for 30 minutes for primary and secondary antibody, were washed 3 times after each stain, and were resuspended in 1% paraformaldehyde overnight until time of read the next morning.

**Cell Scan and Analysis.** Cell marker expression was measured using two-colour detection on a Becton Dickinson FACScan. Raw data were analyzed using FlowJo (version 7) software. Cells were gated to include monocytes and lymphocytes together and results were expressed as a percentage.

**Statistical Methods.** Log-transformed TLR2 expression data were evaluated using the PROC GLM procedure of SAS using the following models:

Model 1  \( Y=μ + \text{AMIR group} + \text{parity} + \text{error} \)

Model 2  \( Y=μ + \text{CMIR group} + \text{parity} + \text{error} \)

Where \( Y= \) log percent of a cell population with the phenotype: TLR2+, TLR2+CD14+, TLR2+BAQ155A+, or TLR2+CD3+

The antilogarithm of GLM output is shown in the figures. A Student's t-test was used to compare H-AMIR and H-CMIR groups for the expression of TLR2, and also to compare Average and Low immune response groups.

**Results**

**AMIR classification.** The Proc GLM model significantly accounted for the variation in TLR2 expression \( (R^2=0.39, p<0.04) \). In addition, Type III sums of squares indicated that AMIR group was approaching significance \( (p<0.06) \), whereas increasing parity was significantly associated with increased TLR2 expression \( (p<0.034) \). The LS Mean for TLR2 expression on mononuclear cells of H-AMIR cows was significantly higher \( (p<0.03) \) than that for L-AMIR cows (Figure 1).

**CMIR classification.** The Proc GLM model was not significant in accounting for the variation in TLR2 expression by CMIR group. Data not shown.

**Comparison of TLR2 expression between High, Average and Low AMIR and CMIR groups.** A Student's t-test of the percentage of mononuclear cells expressing TLR2 between H-AMIR and H-CMIR groups indicated that there was a significant difference \( (p<0.05) \) between H-AMIR and H-CMIR groups, but not significantly different between Average A-AMIR and A-CMIR groups, or Low L-AMIR and L-CMIR groups (Figure 2). Additionally, TLR2 expression was significantly different between H-AMIR and H-CMIR on all white cells combined and monocytes (CD14+), but not T cells (CD3+) and B cells (Figure 3).

**Figure 1.** LS Means of percentage of mononuclear cells expressing TLR2 for cows classified by antibody mediated immune response (AMIR). The x axis represents AMIR Group and the Y axis percent expression on all gated mononuclear cells. Significant differences are indicated by letters \( (*p<0.03) \).

**Figure 2.** Percent of mononuclear cells expressing TLR2 among H-AMIR & H-CMIR, A-AMIR & A-CMIR, and L-AMIR and L-CMIR groups. Significant differences are indicated by letters based on Student’s t test \( (*p<0.05) \).
Figure 3. Percent of mononuclear cells expressing TLR2 by subsets, for H-AMIR (N=6) and H-CMIR (N=9) cows. Cell subsets: all mononuclear cells; CD14+ monocytes; BAQ155A+ B cells; CD3+ T cells. Within a cell subset, means with different letters differ significantly (Student’s t test, *p<0.05).

Discussion

TLRs bind to different types of pathogens by dimerizing with themselves or other TLRs to alert the cell of an infection (Kumar et al. (2011)). For example, TLR2 can dimerize with TLR6 to detect lipotechoic acid (LTA) on Gram+ organisms like Staphylococcus aureus or streptococci known to cause mastitis. TLR2 can also dimerize with TLR1 to bind lipoarabinomannan (LAM), a component of mycobacteria such as Mycobacteria avium ssp. paratuberculosis (MAP) that causes Johne’s disease. Diseases including mastitis are costly for the dairy industry. The cost of mastitis in Canada is estimated to be $200 per case of mastitis, and one out of every five dairy quarters in Canada is infected with a mastitis-causing pathogen at any given point in time (Canadian Bovine Mastitis Research Network, “What’s New in the World of Mastitis Research” http://www.medvet.umontreal.ca/rcrmb/dynaniques/PDF_AN/Results/NewspaperWhatsNew.pdf (2009)).

Recent work by Thompson-Crispi et al. (2013) has shown that H-AMIR animals have significantly less mastitis. Additionally H-AMIR cows tended to have less severe mastitis than L-AMIR cows. In the current study evaluating differences in TLR2 expression on cells among AMIR and CMIR phenotypic groups, cows with H-AMIR had more total mononuclear cells expressing TLR2 compared to L-AMIR cows. As TLR2 is responsible for binding PAMPs associated with pathogens causing mastitis and initiating protective immune responses this may be a possible explanation for better protection in cattle with a H-AMIR phenotype.

This study also looked at the percentage of monocytes, T, and B cells expressing TLR2 among cows that were biased for H-AMIR (N=6) and or H-CMIR (N=9) responses. Work by Kwong et al. (2011) in cattle reported a greater proportion of CD14+ cells among peripheral blood mononuclear cells expressing TLR2. In the current study where animals were categorized into H-AMIR or a H-CMIR phenotype, there was a significantly greater percentage of CD14+ monocytes expressing TLR2 among H-AMIR cows in contrast to H-CMIR cattle. That H-AMIR cows had a higher proportion of CD14+ cells expressing TLR2 may indicate that monocytes of H-AMIR cows are better at detecting pathogens and initiating an immune response.

Though not significant, T cells from H-AMIR cows had a higher expression of TLR2 compared to H-CMIR cows. It has been reported that the expression of TLR2 on bovine and murine γδ T cells can stimulate the production of cytokines that initiate an immune response independent of T-cell receptor (TcR) stimulation (Wesch et al. (2011)). So while the population of T cells evaluated in this study was low and not further defined by their expression of γδ or αβ TcR, the fact that H-AMIR cows expressed more TLR2 may contribute to enhanced innate signalling and the development of a strong antibody response phenotype. Further study to define the T cell population using additional cell surface markers may better reveal differences in TLR2 expression between H-AMIR and H-CMIR groups.

Results from this study also showed no significant difference between the percentage of B cells expressing TLR2 between H-AMIR and H-CMIR groups. Given that B cells are involved in generating an antibody response, there was an expectation to identify a significant difference in the percentage of cells expressing TLR2 between these groups. In this study, the frequency of B cells was low, and cells were not defined by additional staining of surface receptors such as IgM or other markers. Further study to better define B cell populations may help to identify key differences in TLR2 expression between H-AMIR and H-CMIR groups.

This study examined baseline expression of TLR2 on mononuclear cells isolated from the blood without activation by pathogens or molecular structures (ligands) derived from pathogens. Future studies will evaluate differences between AMIR and CMIR response groups before and after stimulation with ligands derived from intracellular (e.g. MAP) and extracellular (e.g. E. coli) pathogens known to cause disease in dairy cattle. Research in this area will help to better elucidate innate mechanisms, such as TLR activation, involved in effectively signalling an adaptive immune response.

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Literature Cited