ABSTRACT: Genetic factors appear to influence resistance to Johnne’s disease (JD). A single nucleotide polymorphism (SNP) located in the promoter of bovine macrophage migration inhibitory factor (MIF) (-298 A>G) for example, was previously found to be associated with Mycobacterium avium subsp. paratuberculosis (Map) infection status. The objective of present study was to determine if this SNP affects bovine macrophage function. In order to evaluate this objective, the gene encoding mCherry was cloned into the clinical isolate of Map, then monocyte-derived macrophages (MDM) from healthy Holstein cows homozygous for genotypes MIF -298 AA (n=6) or GG (n=6) were infected in vitro with this Map isolate to determine MDM effector function. MIF secretion was also assessed in genotype-specific peripheral blood mononuclear cells (PBMC), monocytes and MDM following stimulation. Although MDM phagocytized the reporter Map, and PBMC, monocytes and MDM expressed MIF following stimulation, genotype-specific differences were not detected. 

Keywords: Mycobacterium avium subsp. paratuberculosis (Map); Macrophage migration inhibitory factor (MIF); Single nucleotide polymorphism (SNP)

Introduction

Johnne’s disease (JD) is a chronic enteric disease of ruminants caused by Mycobacterium avium subsp. paratuberculosis (Map). JD is present in all livestock-producing countries, and causes significant economic losses due to decreased productivity, increased susceptibility to other disease, and premature culling (Chioldini et al. (1984)). JD is a slow progressing disease that is not easy to diagnose during early stages of infection after calves are infected with Map.

Host genetic factors influence resistance to JD. Susceptibility to Map infection in cattle is known to be a heritable trait, with heritability estimates ranging from 0.06 to 0.183 (Mortensen et al. (2004)). A previous genetic association study conducted by Verschoor et al. (2011) found an association between a SNP in the promoter of the bovine macrophage migration inhibitory factor (MIF) gene (SNP -298A>G) and Map infection status; a significant additive effect was found between the ‘G’ allele and the likelihood of testing positive for JD based on ELISA.

MIF is an important immunoregulator and plays a critical role in the control of bacterial infections. MIF stimulates the release of pro-inflammatory molecules, neutralizes the anti-inflammatory effects of glucocorticoids, and can suppress cellular apoptosis (Oddo et al. (2005)).

A major constraint with studying host-Map interactions is that it is difficult to detect cells infected with Map, mainly due to the bacteria’s slow growth rate. Fluorescent reporter genes have therefore been introduced into other slow growing Mycobacterium species to overcome this problem (Zafer et al. (2001)). The mCherry reporter DsRed for example, which was initially cloned from the coral Discosoma striata (Carrol et al. (2001)), can be used to identify viable bacteria.

In this study, a reporter Map clinical isolate expressing mCherry was generated and used to determine if bovine MIF (SNP -298 A>G) affects macrophage function. In order to achieve this, monocyte-derived macrophages (MDM) from healthy Holstein cows having either the MIF -298 AA (n=6) or GG genotypes (n=6) were infected in vitro with the mCherry-Map strain. MIF secretion was also assessed in genotype-specific peripheral blood mononuclear cells (PBMC), monocytes and MDM following stimulation with sodium pyrrolidinedithio-carbamate (PDTC).

Materials and Methods

Plasmid (pMADDGPM5) Construction, Amplification and Verification. The mCherry variant of mRFP1 was amplified by PCR from pCHAP6698 (Gross et al. (2000)) using primers BamHI_For1 and Nhel_Rev1 with Pfx Polymerase (Invitrogen). The product was enzymatically digested with BamHI and Nhel (New England Biolabs) and purified using the Gel/PCR DNA Fragment Extraction Kit. The purified insert was cloned into the E. coli-Mycobacteria shuttle vector pKMOP-B by ligation with T4 DNA ligase (Invitrogen) using a 3:1 molar ratio of vector to insert. The ligation reaction was then transformed into E. coli in an electroporation chamber (BioRad) by high voltage electroporation (Dower et al. (1988)). The transformed E. coli was plated onto LB plate containing 50 ug/ml of kanamycin and screened by PCR for the mCherry variant of mRFP1. The verified colony was inoculated and cultured in LB broth containing 50 ug/ml kanamycin. Plasmids were purified using PureYield™ Plasmid Miniprep System.
was monocyte and MIF secretion for 3 days and mediated different and incubated at 37°C with 5% CO₂. The cell suspension was incubated at 37°C for 24 hr, harvested and plated on 7H11 agar containing 50 µg/ml kanamycin. Plates were incubated at 37°C for 4-8 weeks until colonies were visible. Colonies were screened using PCR to detect pMA -hsp65 was used to confirm colonies were Map. Fluorescent mCherry-MAP was visualized with the Zeiss Axiovert 200M microscope using a 590 nm excitation filter.

Animal Genotyping. Blood was collected from the tail vein of 50 healthy Holstein cows. DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen). The tetramer primer ARMS-PCR technique (Ye et al. (2001)) was used to genotype the SNP (-298 A>G) in the promoter of the MIF gene. Six each of homozygous genotypes was selected for the study.

Isolation of PBMC, Monocyte and Culture of MDM. PBMC were isolated using Ficoll-plaque™ Premium column according to the manufacturer’s instructions (Fisher Scientific). PBMC were transferred into T75 flask and incubated at 37°C with 5% CO₂. Adherent monocytes differentiated into mature macrophage (MDM) after 9-14 days and media was replaced every 3 days.

Infect MDM with mCherry-MAP. MDM (5x10⁴/well) were infected with 10:1 ratio of mCherry-MAP for 3 hr, and the fluorescent was measured (excitation/emission 587/610 nm) 3, 24, 48, 72 & 96 hr post infection to measure the phagocytosis and viability.

Stimulation of PBMC, Monocytes and MDM and MIF secretion. 2x10⁵/well of PBMC, or one-day-old monocytes or 5x10⁵/well MDM were stimulated with 100 µM of sodium pyrrolidinedithiocarbamate (PDTC) for 24 & 48 hr. Culture supernatant was collected and MIF secretion was measured using a modified human DuoSet ELISA Development System (R&D System Inc.). The inter- and intra-run CV for MIF-ELISA were 8.3% and 3.6%, respectively.

Statistical Analyses. MIF concentration from PBMC, monocytes and MDM were log transformed prior to analysis in order to stabilize variances. A mixed model, including time, genotype, treatment (infected versus control) and genotype by treatment as fixed effects, and cow by treatment as random effects was used to analyze repeated measurements over time for MDM phagocytosis of Map, viability of MDM and MIF secretion. All calculations were performed using the mixed procedure for SAS (SAS 9.3), and significance was determined at a p-value < 0.05. Values are presented as LSM+SE.

Results and Discussion

The purpose of this study was to determine if the SNP -298 A>G in MIF affects bovine macrophage function, and MIF secretion after cell stimulation. In order to evaluate this objective, a reporter strain of mCherry-Map was generated (Fig. 1). Map is known for very slow growing rate, and contaminating microbes easily outgrow it. To reduce this risk and rapidly detect cells infected with Map, the anti-kanamycin plasmid ligated with mCherry fluorescence was electroporated into the clinical isolate strain GC86. Successful development of this reporter mCherry-Map strain will be invaluable for future JD research, since it’s easily observed under a fluorescent microscope and rapid readouts can be acquired from a plate reader using the 587 nm excitation and 610 nm emission filters.

Figure 1: Microscopy of mCherry Map GC86

Figure 2: In vitro MDM infection with mCherry-Map
MDM from MIF -298 AA (n=6) or GG genotype (n=6) cows were co-cultured in vitro with mCherry-MAP for 3 hr to allow for phagocytosis (Fig. 2), and then continuously cultured until 96 hr post infection to assess Map and MDM viability. There was no difference in mCherry fluorescence from the AA and GG genotypes over the observation period. The infected MDMs were also examined microscopically to confirm phagocytosis of Map (Fig. 2). MDM viability was assessed using the Calcein AM stain, and treatment and genotype differences were not detected.

Figure 3: Phagocytic uptake of Map by bovine MDM

In order to functionally assess the effect of MIF SNP (-298 A>G) on MIF secretion, genotype-specific PBMC, monocytes and MDM were stimulated with PDTC, and ELISA was used to measure MIF secretion. MIF secretion was greatest from PBMC (Fig. 3 A), and lymphocytes were likely the primary source of MIF based on the low level of secretion observed from monocytes (Fig. 3B). Interestingly, MIF secretion greatly increased as monocytes differentiated into MDM (Fig. 3C). MIF secretion from PBMCs, monocyte and MDM was not significantly different across the MIF SNP (-298 A>G) genotypes.

Figure 4: MIF expression from (A) PBMC, (B) bovine monocytes and (C) MDM activated with PDTC.

Conclusion

A mCherry fluorescence reporter strain of Map (mCherry-MAP) was generated. This reporter MAP can be used to improve our understanding of the mechanisms involved in JD pathogenesis, genetic susceptibility, and host-pathogen interaction. In this study, analyses of the MIF SNP (-298 A>G) did not reveal genotype differences in macrophage function as indicated by in vitro phagocytosis of Map and MDM cell viability, as well as MIF secretion by PBMC, monocytes and MDM. Since MIF is a multifunctional cytokine that is stored within intracellular pools before secretion, it is possible that genotype effects may have been overlooked using functional endpoints assessed in this study. It is also possible however, that previously identified genotype associations with Map infection status may be due to linkage to a neighboring putative gene.

Literature Cited


