Expression of interleukin-10 and suppressor of cytokine signaling-3 associated with susceptibility of cattle to infection with *Mycobacterium avium* subsp *paratuberculosis*

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**Objective**—To determine functional characteristics of monocytes obtained from cows with subclinical infection with *Mycobacterium avium* subsp paratuberculosis (MAP) that may have predisposed those cows to becoming infected with MAP.

**Sample Population**—Monocytes obtained from 5 uninfected cows and 5 cows subclinically infected with MAP in a herd with a high prevalence of paratuberculosis (ie, Johne’s disease).

**Procedures**—Monocytes from uninfected and subclinically infected cows were incubated with MAP for 2, 6, 24, 72, or 96 hours. Variables measured included expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-10, IL-12, transforming growth factor-β, and suppressor of cytokine signaling-3 (SOCS-3); apoptosis of monocytes; acidification of phagosomes; and killing of MAP.

**Results**—Monocytes from infected cows had greater expression of IL-10 and SOCS-3 at 2 hours of co-incubation with MAP and lower expression of TNF-α and IL-12 when results for all incubation times were combined. Monocytes from infected cows had a greater capacity to acidify phagosomes. No differences were observed in the rate of apoptosis or capacity of monocytes to kill MAP organisms.

**Conclusions and Clinical Relevance**—Monocytes obtained from cows with subclinical infection with MAP had upregulated expression of IL-10 and SOCS-3 within the first 2 hours after exposure to MAP organisms. Although this did not inhibit acidification of phagosomes, apoptosis of monocytes, or attenuation of the capacity to kill MAP organisms, it may have attenuated the capacity of mononuclear phagocytes to initiate inflammatory and adaptive immune responses.


Paratuberculosis (ie, Johne’s disease) is a chronic granulomatous enteritis of wild and domestic ruminants that is caused by infection with *Mycobacterium avium* subsp *paratuberculosis* (MAP). Not all exposed cattle become chronically infected. This may be attributable in part to the number of organisms ingested or age at which cattle are exposed. However, even some cattle experimentally infected with large numbers of organisms recover from the infection. The role of genetic variation in resistance to paratuberculosis is uncertain. Analysis of results of studies suggests that paratuberculosis is heritable. Heritability estimates for susceptibility to MAP infection in vaccinated cattle is 0.09, which is equivalent to heritability estimates for other diseases considered to have a polygenic mode of inheritance.

The incidence of paratuberculosis reportedly varies among breeds of cattle. There is a disproportionately high incidence of infection in Shorthorn breeds in Minnesota; the province of Ontario, Canada; and England. There is also a higher incidence in Channel Island breeds in England. Reportedly, the incidence of infection in Jersey cows is 4.78%, whereas Holstein cows have an infection rate of 0.27%. Because herd size, management practices, and exposure to wild animals that carry the organism may have contributed to these differences, a definitive breed predisposition cannot be inferred from the results of these studies.

Studies in other species have established a link between susceptibility to microbial infection and genotypic variation of the host. In most instances, susceptibility is controlled by multiple genes. The most studied associations are for major histocompatibility class (MHC) II and solute carrier family 11 member A1 (formerly termed natural resistance macrophage protein 1). Polymorphisms of MHC II and solute carrier family 11 member A1 have been associated with susceptibility to *Mycobacterium tuberculosis*. Other gene polymorphisms associated with susceptibility to *M. tuberculosis* include the receptor antagonist to interleukin (IL)-1, transporter associated with antigen processing, tumor necrosis factor (TNF)-α and -β, vitamin D receptor, and mannose-binding lectin.

In the study reported here, we investigated differences in the response of bovine monocytes to infection with MAP in vitro. To identify functional responses that may predict genetic susceptibility to MAP infection, we compared responses of monocytes from cows chronically infected with MAP to those obtained from uninfected cows of the same highly infected herd. It is probable that control cows were exposed to MAP organisms but failed to become chronically infected. Therefore, this group may have represented a relative-
ly resistant population. Because the type of genetic defect could not be predicted, multiple functional responses were evaluated. These included expression of cytokines, including IL-10, IL-12, TNF-α, transforming growth factor-β (TGF-β), and suppressor of cytokine signaling-3 (SOCS-3); acidification of phagosomes; apoptosis of macrophages; and ingestion and killing of MAP.

Materials and Methods

Animals—Ten adult Holstein cows were purchased from a herd consisting of 350 cows with a high incidence of paratuberculosis. Cows used in the study were between 5.5 and 7.4 years of age, and all cows were not lactating or pregnant at the time of evaluation. Approximately 25% of the 350 cows in the herd were serologically positive for paratuberculosis, and 10% to 15% had positive results for microbial culture of fecal samples. Mycobacterium avium subsp paratuberculosis organisms had repeatedly been cultured from samples obtained from calf pens, walkways, feed, and soil on this farm; therefore, it was likely that cattle were repeatedly exposed to MAP organisms. All cows had been tested for paratuberculosis 4 times for the preceding 4 years by use of microbial culture of fecal samples and an ELISA. Five of the cows were classified as high fecal shedders (ie, > 100 colonies/g of feces), and the other 5 cows consistently had negative results for serologic tests and microbial culture of fecal samples. None of the cows was clinically ill at the time of purchase. All 10 cows were tested for MAP infection by use of microbial culture of fecal samples, ELISA of serum samples, and measurement of cell-mediated immune response. At the end of the study, all cows were euthanized by administration of an overdose of barbiturate. During necropsy, at least 20 pieces of ileal tissue were collected and submitted to the Minnesota Veterinary Diagnostic Laboratory for mycobacterial culture, and tissue homogenates were used for detection of MAP by use of a polymerase chain reaction (PCR) assay. All test results were positive for infected cows and negative for uninjected cows.

Isolation and culture of bovine monocytes—Peripheral blood mononuclear cells were isolated by centrifugation with a density gradient6 (59%), as described elsewhere. Isolated cells were washed in Dulbecco PBS solution and resuspended at a concentration of 3 × 10^7 mononuclear cells/ml in RPMI medium containing 10% fetal bovine serum. Mononuclear cells (3 × 10^6) were incubated for 90 minutes to allow adherence to 60-mm tissue culture plates or 24-well tissue culture microplates. Nonadhered cells were removed by repeated washing with RPMI medium (37°C), and plates were cultured (6 hours at 37°C and 5% carbon dioxide) in RPMI medium supplemented with 10% fetal bovine serum.

Preparation of MAP—Mycobacterium avium subsp paratuberculosis strain 058 was cultured from samples submitted to the Minnesota Animal Health Diagnostic Laboratory that were obtained from an animal with naturally occurring paratuberculosis. The organism was isolated during the 3-month period preceding use and was confirmed to be MAP on the basis of dependence on mycobactin J for growth and detection of species-specific DNA sequences by use of a PCR assay. The organisms were grown to a concentration of approximately 1 × 10^9/ml; washed, and resuspended in broth, Tween 80, and mycobactin J. Viability at the time of addition to macrophage cultures varied between 81% and 93%. Immediately before use, organisms were washed and resuspended in culture medium.

Semi quantitative reverse transcriptase-PCR assay—A semi quantitative reverse transcriptase (RT)-PCR method with limited amplification of a specific cDNA product was used to characterize expression of TNF-α, IL-10, IL-12, TGF-β, and SOCS-3. These molecules were chosen because of their key roles in the induction of inflammatory and immune responses or because their expression can be altered by mycobacterial infection.12,13 Genomic DNA was removed from mRNA before synthesis of cDNA by use of a commercially available kit. 14 The RT-PCR reaction contained 1X PCR buffer, 0.2mM of each deoxynucleoside triphosphate, 1 µM of each of several specific forward and reverse primers, 0.4 µCi of [32P] d-cytidine triphosphate (3,000 Ci/mmol), 1.25 units of polymerase, and 2 µl of first-strand cDNA template. Primers for these molecules have been described elsewhere.15,16 Analysis of results for multiple dilutions of input cDNA template revealed that for these conditions, the amount of cDNA product directly correlated with the amount of cDNA template (data not shown). Furthermore, all cDNA products were initially analyzed at 20, 23, and 25 cycles to document that product accumulation was still in the exponential phase of the reaction (data not shown). Tumor necrosis factor-α, IL-10, IL-12, TGF-β, and SOCS-3 were amplified for 25, 23, 24, 20, and 26 cycles, respectively. The products were separated on 5% nondenaturing acrylamide gels, and specific products were detected by use of a phosphomaging system. Expression of a control gene (ie, glyceraldehyde-3-phosphate dehydrogenase) was used to standardize the amount of cDNA product in each sample. Intensity of green and red fluorescence was sequentially evaluated immediately by use of a confocal microscope. After incubation for 72 hours, monolayers were washed twice in Dulbecco PBS solution and then lysed by incubation with 0.1% deoxycholate for 5 minutes. The lysate was incubated with a mixture (1:1) of a green fluorescent dye and a mixture (1:1) of a red fluorescent dye. Coverslips were incubated with labeled mycobacteria and coverslips were incubated with labeled mycobacteria. The stain is taken up by acidified phagosomes, and specific products were detected by use of a confocal microscope. Ingestion and intracellular survival of mycobacterial organisms—Monocytes isolated on coverslips were stained with Ziehl-Neelsen carbolfuchsin stain that stains mycobacterial and other acid-fast organisms. The percentage of monocytes containing organisms was determined by counting a minimum of 200 cells by use of light microscopy. Killing of organisms was assessed by use of a live-dead staining kit. After incubation for 72 hours, monolayers were washed twice in Dulbecco PBS solution and then lysed by incubation with 0.1% deoxycholate for 5 minutes. The lysate was incubated with a mixture (1:1) of a green fluorescent stain and propidium iodide stain. Cells were placed on a microscope slide and examined under a microscope with a 10X objective. For this method, live organisms had green fluorescence and dead organisms had red fluorescence. At least 100 organisms were counted, and the percentage of live organisms was determined.

Acidification of phagosomes—Acidification of phagosomes was determined by use of a fluorescence technique with a confocal microscope.12,13 Organisms were labeled with fluorescein isothiocyanate before they were added to macrophage cultures. Bovine monocytes grown on 2 2-cm coverslips were incubated with labeled mycobacteria (10 bacilli/monocyte) for 2 or 6 hours. A red stain (final concentration, 50nM) was added during the last 30 minutes of incubation obtained from an animal with naturally occurring paratuberculosis. The stain is modified to become fluorescent. After incubation, coverslips were washed, inverted onto glass slides, and evaluated immediately by use of a confocal microscope. Intensity of green and red fluorescence was sequentially evaluated immediately by use of a confocal microscope.
recorded at increments of 0.3µM throughout the depth of the cells. Data were analyzed by use of an image-analysis program. Sequential images were merged, and intensity of green and red fluorescence for at least 200 phagosomes containing mycobacteria was quantified; results were reported as a colocalization coefficient. The colocalization coefficient was defined as the density of red fluorescence divided by the density of green fluorescence. Phagosomes in which there were approximately equal amounts of red and green fluorescence appeared yellow in color.

**Apoptosis**—Apoptosis of bovine monocytes was evaluated by use of a staining technique. Other methods to detect apoptosis were not used because of technical problems with the assays. Annexin V and the terminal deoxynucleotidyl-transferase-mediated d-uridine triphosphate-biotin nick end-labeling assay reportedly yield inconsistent results when used to detect apoptosis in bovine macrophages. Bovine monocytes were incubated with and without MAP organisms (10 bacilli/monocyte) for 24 or 48 hours. Monocytes were incubated with staurosporine (500nM) for 6 hours to serve (10 bacilli/monocyte) for 24 or 48 hours. Monocytes were used to detect apoptosis in bovine macrophages. Bovine monocytes were incubated with and without MAP organisms (10 bacilli/monocyte) for 24 or 48 hours. Monocytes were incubated with staurosporine (500nM) for 6 hours to serve as a positive control sample. Then, culture plates were incubated with a stain for 10 minutes, as described elsewhere. Incubation with staurosporine (500nM) for 6 hours to serve (10 bacilli/monocyte) for 24 or 48 hours. Monocytes were used to detect apoptosis in bovine macrophages. Bovine monocytes were incubated with and without MAP organisms (10 bacilli/monocyte) for 24 or 48 hours. Monocytes were incubated with staurosporine (500nM) for 6 hours to serve as a positive control sample. Then, culture plates were incubated with a stain for 10 minutes, as described elsewhere. Cultures were examined by use of a fluorescence microscope with excitation at 350 nm and emission at 461 nm. At least 200 cells were counted, and the percentage of fluorescent nuclei was determined.

**Statistical analyses**—Experiments were conducted in duplicate or triplicate, and mean values were calculated. For all data, differences between monocyte cultures derived from infected and uninfected cows were compared by use of a paired t test in a computerized statistical program. Values of \( P < 0.05 \) were considered to be significant.

**Results**

**Uptake and killing of organisms**—Greater than 90% of macrophages from infected and uninfected cows ingested MAP organisms during the 90-minute incubation period (Table 1). Values did not differ significantly between infected and uninfected cows. Macrophages from infected and uninfected cows failed to kill MAP organisms by 72 or 96 hours after onset of incubation.

**Cytokine expression**—Expression of IL-10, IL-12, TNF-α, TGF-β, and SOCS-3 by monocytes from infected and uninfected cows was evaluated by use of an RT-PCR assay at 2, 6, and 24 hours after addition of the organism to cultures. Expression of IL-10 was significantly \((P = 0.01)\) greater for monocytes from infected cows than for monocytes from uninfected cows at 2 hours but not at other time points (Figure 1). Although monocytes from all 5 infected cows had values that were greater than that of the highest value for monocytes from uninfected cows, the magnitude of increase varied. This resulted in a large SD at the 2- and 6-hour time points.

Results for IL-12 and TNF-α were similar (Figures 2 and 3). When compared with values for uninfected monocytes, values for infected monocytes from infected and uninfected cows had increased expression at 2, 6, and 24 hours after exposure to MAP organisms. Monocytes from uninfected cows had greater mean values at all time points; however, the values did not differ significantly. This was attributable to considerable variation between cytokine expression among the infected cows, as indicated by a high SD. When data for all time points were combined, monocytes for the infected group had significantly \((P = 0.01)\) lower expression of IL-12 and TNF-α.

Expression of TGF-β was significantly greater than baseline values at 2, 6, and 24 hours after onset of incubation in monocytes from infected and uninfected cows (Figure 4). However, we did not detect significant differences in expression between monocytes from infected and uninfected cows.

Expression of SOCS-3 was significantly greater than baseline values at 2, 6, and 24 hours after onset of incubation in monocytes from infected cows and at 6 and 24 hours in monocytes from uninfected cows (Figure 5). Expression increased progressively over time. Values for monocytes from infected cows were significantly \((P = 0.01)\) higher (>-4-fold greater) than values for monocytes from uninfected cows at 2 hours after addition of organisms.

**Acidification of phagosomes**—The capacity of monocytes to acidify phagosomes containing MAP organisms was evaluated 2 and 6 hours after onset of coinubcation. By 2 hours after onset of coinubcation, most phagosomes appeared green in color and the colocalization coefficient was low (Table 1). Responses of monocytes from infected and uninfected cows were similar. By 6 hours after onset of coinubcation, treated and untreated cultures had a substantial increase in colocalization coefficients, but colocalization coefficients for monocytes from in-

### Table 1—Mean ± SD responses of monocytes derived from uninfected cows and cows with subclinical paratuberculosis incubated with or without Mycobacterium avium subsp paratuberculosis (MAP) organisms.

<table>
<thead>
<tr>
<th>Test</th>
<th>Incubation time (h)</th>
<th>MAP</th>
<th>Uninfected cows</th>
<th>Infected cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis (%)</td>
<td>24</td>
<td>–</td>
<td>11 ± 2</td>
<td>14 ± 3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>+</td>
<td>21 ± 4</td>
<td>17 ± 5</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>–</td>
<td>18 ± 5</td>
<td>22 ± 6</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>+</td>
<td>28 ± 5</td>
<td>27 ± 7</td>
</tr>
<tr>
<td>Phagosome acidification</td>
<td>2</td>
<td>+</td>
<td>0.27 ± 0.11</td>
<td>0.30 ± 0.15</td>
</tr>
<tr>
<td>(colocalization coefficient)</td>
<td>6</td>
<td>+</td>
<td>0.48 ± 0.16</td>
<td>1.13 ± 0.23*</td>
</tr>
<tr>
<td>MAP uptake (% of monocytes)</td>
<td>1.5</td>
<td>+</td>
<td>92 ± 4</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>MAP survival (% alive)</td>
<td>96</td>
<td>+</td>
<td>115 ± 11</td>
<td>122 ± 9</td>
</tr>
</tbody>
</table>

*Within a row, value differs significantly \((P = 0.01)\) from the value for uninfected cows. – = Not incubated with MAP organisms. + = Incubated with MAP organisms.
infected cows were greater than those for monocytes from uninfected cows.

Apoptosis—Apoptosis was evaluated 24 and 48 hours after addition of MAP organisms. Mean ± SD percentage of apoptotic cells in negative control cultures 24 and 48 hours after onset of incubation was 11 ± 3% and 14 ± 6%, respectively. Mean percentage of apoptotic cells in the positive control sample was 26 ± 5%. The percentage of apoptotic monocytes for infected cows did not differ significantly from that for uninfected cows (Table 1).

Figure 1—Mean ± SD expression of interleukin (IL)-10 by bovine monocytes derived from 5 cows with subclinical paratuberculosis (black bars) or 5 uninfected cows (stippled bars) in response to ingestion of Mycobacterium avium subspecies paratuberculosis (MAP) after incubation for various periods of time. Values reported represent the mean band density for IL-10 after adjustment for the band density of a control gene (i.e., glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). *Within a time point, value differs significantly (P < 0.05) from the value for monocytes from uninfected cows. Time 0 = Time at which MAP organisms were added to monocyte cultures.

Figure 2—Mean ± SD expression of IL-12 by bovine monocytes derived from cows with subclinical paratuberculosis (black bars) or 5 uninfected cows (stippled bars) in response to ingestion of MAP after incubation for various periods of time. Values reported represent the mean band density for IL-12 after adjustment for the band density of a control gene (i.e., GAPDH). When data from all time points were combined, monocytes from infected cows had significantly (P < 0.05) less IL-12 expression, compared with expression for monocytes from uninfected cows. See Figure 1 for remainder of key.

Figure 3—Mean ± SD expression of tumor necrosis factor-α (TNF-α) by bovine monocytes derived from cows with subclinical paratuberculosis (black bars) or 5 uninfected cows (stippled bars) in response to ingestion of MAP after incubation for various periods of time. Values reported represent the mean band density for TNF-α after adjustment for the band density of a control gene (i.e., GAPDH). When data from all time points were combined, monocytes from infected cows had significantly (P < 0.05) less TNF-α expression, compared with expression for monocytes from uninfected cows. See Figure 1 for remainder of key.

Figure 4—Mean ± SD expression of transforming growth factor-β (TGF-β) by bovine monocytes derived from cows with subclinical paratuberculosis (black bars) or 5 uninfected cows (stippled bars) in response to ingestion of MAP after incubation for various periods of time. Values reported represent the mean band density for TGF-β after adjustment for the band density of a control gene (i.e., GAPDH). Values did not differ significantly between monocytes from infected and uninfected cows. See Figure 1 for remainder of key.
Discussion

Studies13,14 have established a link between susceptibility to mycobacterial infection and genotypic variation of the host. These defects have been associated with a variation in antimicrobial mechanisms within macrophages or the capacity of macrophages to induce a T-helper (Th)1-type adaptive immune response.15-18 In the study reported here, we compared responses of monocytes from cows with subclinical paratuberculosis with those for monocytes from uninfected cows in an attempt to identify functional differences that would increase susceptibility to MAP infection. Because MAP organisms had been consistently isolated from the environment (calf and cow barns) and feed for this herd, it is likely that uninfected cows were repeatedly exposed to MAP organisms. Therefore, they may have represented a population that was relatively resistant to MAP. However, we cannot eliminate the possibility that uninfected cows were exposed to a smaller number of organisms or were exposed at an older age and therefore were able to avoid becoming chronically infected.

Given the aforementioned limitations, analysis of our results indicated that monocytes from infected cows had more than a 4-fold increase in IL-10 expression within 2 hours after onset of incubation with MAP, whereas monocytes from uninfected cows had only a slight increase. Interleukin-10 is an immunomodulatory cytokine produced by various cells, including monocytes or macrophages and T and B lymphocytes.33 This cytokine plays a pivotal role in immune responses by enhancing Th2-type immune responses and suppressing Th1-type immune responses.26-28 Interleukin-10 is believed to suppress Th1-type immune responses largely through inhibition of the production of IL-12 by macrophages.29,30 Interleukin-10 also inhibits expression of proinflammatory cytokines and antigen presentation by macrophages and dendritic cells.29,30 Additionally, IL-10 in combination with TGF-β is involved in suppressing T-cell responses and inducing tolerance.28,31-35 Because induction of a Th1-type immune response is essential for resistance to mycobacterial infection, early production of IL-10 may be conducive to establishment of a chronic infection.

Monocytes from infected cows also upregulated SOCS-3 expression 2 hours after addition of MAP to cultures, whereas there was no upregulation for monocytes from uninfected cows. Suppressors of cytokine signaling are a family of molecules that inhibit expression of a wide range of cytokines, including interferon (IFN)-γ and IL-12, by binding to and inhibiting Janus kinases and cytokine receptors.31 Inhibitory effects of IL-10 on inflammatory responses of monocytes or macrophages appear to be largely dependent on the capacity to induce de novo synthesis of SOCS-3.30 Therefore, early expression of IL-10 by monocytes from infected cows may have induced SOCS-3 expression, thereby blunting their capacity to initiate inflammatory and immune responses. This hypothesis is supported by the concurrent decrease in total IL-12 and TNF-α expression by monocytes in the study reported here.

Other results support a role of IL-10 in susceptibility to MAP infection. In 1 study,19 bovine macrophages incubated with MAP produced relatively large amounts of IL-10, compared with the amount produced by macrophages incubated with IFN-γ and lipopolysaccharide. Inhibition of IL-10 activity, documented by the addition of a neutralizing anti–IL-10 antibody, increased expression of TNF-α, IL-12, IL-8, and MHC II by bovine monocyte-derived macrophages incubated with MAP organisms.31 When bovine monocyte-derived macrophages were incubated with MAP or the less pathogenic M. avium subsp. avium, macrophages phagocytizing MAP organisms had greater expression of IL-10 and lesser expression of TNF-α.32 Macrophages phagocytizing MAP organisms also had a reduced capacity to increase expression of MHC I and II after exposure to IFN-γ.33 Several in vitro studies33-36 have documented that MAP organisms are ingested by bovine monocytes and monocyte-derived macrophages but are not killed. Cytokines appear to modulate killing of organisms by macrophages. Prior treatment of bovine monocytes with IFN-γ, granulocyte macrophage colony–stimulating factor, or high doses of TNF-α restricts growth of MAP in vitro.34,35 Addition of a neutralizing anti–IL-10 antibody results in a noticeable improvement in the capacity of macrophages to kill MAP organisms.35,36 This increased killing is associated with increases in the rate of monocyte apoptosis, acidification of phagosomes, and production of nitric oxide.34,35 Alternatively, in the study reported here, major differences in apoptosis, acidification of phagosomes, or killing of organisms were not detected despite a greater expression of IL-10 by monocytes derived from infected cows.

Analysis of results of the study reported here indicated that monocytes from cows with subclinical paratuberculosis rapidly upregulated expression of IL-10 and SOCS-3 within 2 hours after ingesting MAP
organisms and attenuated expression of IL-12 and TNF-α. Alternatively, specific anticytobacterial defects were not identified. Early suppression of monocyte activation by IL-10 and SOCS-3 combined with a decrease in IL-12 expression would be expected to attenuate induction of a Th1-type immune response. Because IL-10 can stimulate SOCS-3 transcription and suppress IL-12 transcription, dysregulation of IL-10 transcription could be a primary factor in susceptibility to MAP infection. Although these differences could have been the result of the infection and therefore were not determined genetically, additional evaluation of genotypic variation in the regulation of IL-10 expression is warranted.

References


