



Increased blood-circulating interferon- γ , interleukin-17, and osteopontin levels in bovine paratuberculosis

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ABSTRACT

Paratuberculosis-infected cattle initially develop an effective cell-mediated immune response that declines as the disease progresses. Blood is one of best sources for characterizing the inflammatory status of infected cows and for studying mediators related to chronic diseases. The aim of this study was to evaluate the cow-level association between blood cytokine concentration, the influence of serum on immune cell proliferation, and dairy cows naturally infected with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Positive animals ($n = 41$) from 19 herds were selected on the basis of 2 positive fecal culture results and divided into 2 groups: single-positive, or serum ELISA-negative cows ($n = 32$), and double-positive, or cows that gave positive results for both mycobacterial culture and serum ELISA ($n = 9$). Negative animals ($n = 39$) were selected from paratuberculosis-negative herds in which at least 80% of the animals had been diagnosed as negative by fecal culture and ELISA and that did not produce positive results during the 2-yr study. Analysis of plasma levels of the cytokines IL-4, IL-10, IL-17, IFN- γ , and osteopontin was performed, revealing distinct patterns. The ELISA-positive cows with MAP shedding had similar plasma concentrations of IL-4 and IL-10 but elevated levels of IFN- γ , IL-17, and osteopontin, which is indicative of inflammatory disease in these subclinical positive cows. In vitro MAP infection of bovine macrophages showed increased gene expression of tumor necrosis factor- α , IL-1 β , IL-6, IL-23, and transforming growth factor- β as early as 6 h postinfection for all of the cytokines involved in the establishment of a T-helper type-17 immune response. To determine the systemic influence of serum on immune cell functions, lymphoproliferation assays were also performed in presence of JD serum. The serum from shedding cows showed 15% less pro-

liferation. These results indicate that infected cows have a lower systemic capacity to maintain a protective immune response and that, as the disease progresses, an emerging T-helper type-17 immune response is established.

Key words: bovine paratuberculosis, interleukin-17, osteopontin, cell proliferation

INTRODUCTION

Bovine paratuberculosis, also called Johne's disease (JD), is caused by infection with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Infection by this pathogen results in a chronic, slowly progressive granulomatous enteritis affecting a wide range of wildlife and domesticated ruminants (Collins et al., 1994; Carta et al., 2013). The MAP pathogen is an intracellular bacterium that infects mainly the immune cells associated with the digestive system. The mycobacteria establish themselves in the intestinal submucosa. *Mycobacterium avium* ssp. *paratuberculosis* survives by infecting, residing, and slowly proliferating in macrophages or dendritic cells as an intracellular parasite. The onset of clinical disease is unpredictable. Several years after the initial infection (5–7 yr), the animal will develop clinical signs of the disease, which include intermittent to chronic diarrhea, granulomatous inflammation, and cachexia, and which cause the eventual death of the infected animal. The disease is insidious for several reasons: infected animals excrete the pathogen in feces before the clinical signs appear, shedding may be intermittent, and only some infected animals show a humoral immune response characterized by the circulation of MAP-specific antibodies in the blood (Nielsen and Toft, 2006). Efforts to control JD have been impeded by the failure to detect early infection, the lack of an effective vaccine (Park et al., 2011; Stabel et al., 2011; Alonso-Hearn et al., 2012; Knust et al., 2013; Lu et al., 2013), the confounded results by cross-reactivity due to homology between mycobacterial species (Stabel et al., 2013), and the lack

Received May 23, 2013.

Accepted February 12, 2014.

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of sensitive and specific diagnostic tests (Sockett et al., 1992; Whitlock et al., 2000; McKenna et al., 2005).

Discrepancies in the results of studies involving assays to measure cell-mediated immunity can be attributed in part to the fact that these immune responses occur early in infection. Over time, persistent infection triggers an event that dysregulates the immune response and allows the disease to progress. During the early molecular events of the infection, MAP subverts the defenses of the specialized antigen-presenting cells (Demangel and Britton, 2000). The pathogen blocks phagosomal acidification after ingestion by phagocytic cells (Deretic et al., 1997; Astarie-Dequeker et al., 1999; Weiss et al., 2005). Infection also results in downregulation of expression of major histocompatibility complex I and II (Weiss et al., 2001), which is needed to activate an efficient adaptive response against MAP. The mycobacterium is thought to alter responses mediated by the classical toll-like receptors (TLR)-1 and TLR4 (Subharat et al., 2012), as well as TLR9, (Arsenault et al., 2013) that are required for its detection by the immune cells. The immune response breakdown also appears to involve dysregulation of macrophage or dendritic cell control of the cytokine network (Cooper and Khader, 2008). The network is targeted by mycobacteria, starting with antigen-presenting cells, which play a pivotal role in shaping the innate and adaptive immune response. The pathogen seems to interfere in many ways with cytokine expression and response, thereby ensuring its increased survival and tolerance in the host (Allen et al., 2009). Such changes in the immune response may make it very difficult for the host to fight the pathogen and may eventually lead to immune evasion by MAP and ultimately a misdirection of the immune response.

Analysis of the inflammatory profile of MAP-infected cows seems to be a fundamental step toward a better understanding of immune status as the disease progresses. In the early stages of MAP infection, it is generally accepted that mycobacteria elicit a cell-mediated response by the host, supported by an initial T-helper type-1 (T_{H1}) immune response that is mediated by IFN- γ . However, it is not clear how the immune system copes with this pathogen as the disease progresses. Immunological studies in subclinical adult ruminants led to the now-controversial proposal that the T_{H1} -dominant lymphocyte immune response (IFN- γ secretion) is induced predominantly during the early subclinical stages of the infection and wanes later on, followed by a switch to a T-helper type-2 (T_{H2}) response (Chiodini et al., 1984; Stabel, 2000; Coussens, 2004). The T_{H1}/T_{H2} paradigm was established mainly from the results of early classical mycobacterial infection experiments and animal disease models for fungal and *Leishmania* infec-

tions (reviewed in Spellberg and Edwards, 2001). In the last few years, however, major discrepancies have been found in this model (Gor et al., 2003; Abebe and Bjune, 2009; Begg et al., 2011). The hypothesized switch has now been supplanted by other hypotheses supported by accumulating circumstantial evidence. Accordingly, many researchers have suggested that the antiinflammatory cytokine IL-10 would account for the loss of proinflammatory responses (Buza et al., 2004; Coussens et al., 2004). In particular, data from *Mycobacterium tuberculosis* studies have provided evidence for the role of IL-10 in the regulation of phagosome maturation in macrophages (Via et al., 1998). The capacity of IL-10 to impair mycobacterial phagosome maturation was confirmed in MAP-infected bovine macrophages (Weiss et al., 2005). Recently, a potential link with the IL-10-secreting regulatory T lymphocytes (T_{reg}) was proposed. The T_{reg} population would develop during the subclinical phase of MAP infection and progressively limit the peripheral and tissue-specific T_{H1} immune responses of JD-positive cows (de Almeida et al., 2008; Coussens et al., 2012). This T_{H1}/T_{H2} concept has been challenged by the description of tolerizing T_{reg} and by proinflammatory T-helper type-17 (T_{H17}) cells, a novel T-cell population characterized by the master transcription factor ROR γ , the surface markers IL23R, and by production of the proinflammatory IL-17 cytokine, among others. In the last few years, several human autoimmune and inflammatory disorders, such as inflammatory bowel disease, rheumatoid arthritis, allergic asthma, and psoriasis (Varona et al., 2003; Teraki et al., 2004; Lundy et al., 2005; Hirota et al., 2007), have been associated with the secretion of IL-17 by T_{H17} lymphocytes. This subset of T cells requires both pro- and antiinflammatory cytokines such as IL-1 β , IL-6, and transforming growth factor- β (TGF- β) to support their differentiation. The proinflammatory cytokine IL-17 is believed to sustain a long-term inflammation site, causing dysfunction of a tissue that has been invaded with numerous immune cells. Interesting data also suggest that the T_{H17} subset may be involved in Crohn's disease (Brand, 2009). It remains unknown whether this subset has implications in JD, but some recent studies proposed a role in mycobacterial host defense (Curtis and Way, 2009). Also, it has been shown that IL-17 is upregulated in mesenteric lymph node cells from MAP-infected cows (Shu et al., 2011). Besides the similarities between JD and Crohn's disease, the implication of the T_{H17} population in inflammatory bowel disease provides insight into the potential involvement of this lymphocyte class in the development of paratuberculosis. Osteopontin is implicated in both chronic inflammation (Agniholt et al., 2007) and T_{H17} differentiation pathway (Chen et al., 2010) and is associated with Crohn's disease (Glas et

al., 2011). Therefore, it could be interesting to establish whether or not this cytokine is associated with paratuberculosis, a chronic enteric infection. Modulation of cytokine signaling pathways by MAP could alter the secretion of cytokines and their effects on the differentiation and function of effector T cells. Given that different hypotheses have been advanced to explain regulation of the host immune response, identification of cytokines secreted as the disease progresses could also shed light on this complex disease.

With a view to improving our understanding of the mechanisms involved in the development of JD, the goals of the present study were to investigate the circulating inflammatory status of JD-positive cows by analyzing some blood cytokines and by evaluating the lymphoproliferative capacity of serum collected from MAP-infected cows. We hypothesized, first, that bovine paratuberculosis induces an anergic state, reducing the capacity of the host immune response, and second, that a progressive shift toward a chronic inflammation status is established over the lengthy course of subclinical MAP infection.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were carried out according to the Canadian Council on Animal Care guidelines for institutional animal use, and ethical approval for the study was obtained from the Agriculture and Agri-Food Canada Animal Ethics Committee (protocol number 362).

Animal Diagnosis and Selection

The animals used for this study were screened for paratuberculosis by the Laboratoire d'épidémiosurveillance animale du Québec (Saint-Hyacinthe, QC, Canada), both by bacterial culture of individual fecal samples (fecal culture, **FC**) and by serological detection using the Pourquier ELISA (Idexx Laboratories, Markham, ON, Canada) as described by the manufacturer. A total of 24 tiestall dairy herds in Quebec with known status (19 infected and 5 uninfected) were sampled twice in 2011. Negative cows (−/−; $n = 39$) were selected from the 5 uninfected herds. In those herds, at least 80% of all the cows had been confirmed negative in 2 consecutive bacterial FC. All the animals in the negative herds were also found to be negative by serological detection. These animals were older than 4.5 yr and were tested again by FC on the day of sampling to ensure that no positive animals would be used in the subsequent experiments. A total of 41 animals were identified

positive by FC from the 19 positive herds. For further analysis, these shedding animals were separated into 2 groups based on serum ELISA results. The first group was called single-positive (+/−; $n = 32$) and included cows that received a positive diagnosis by FC and were negative by serum ELISA; the second group was called double-positive (+/+; $n = 9$) and included those cows diagnosed as MAP-positive by both FC and ELISA.

Blood Collection and Treatment

Blood samples were taken from the jugular vein in 10-mL Vacutainer tubes (BD Biosciences, Mississauga, ON, Canada) with EDTA for plasma or without anticoagulant for serum. The EDTA tubes were chilled on ice immediately after sampling, whereas the serum was allowed to clot at 4°C for 4 h. The samples were centrifuged at $1,800 \times g$ and 4°C for 15 min and then stored at −80°C into 2-mL aliquots until analysis.

Proliferation Assay

The capacity of the serum to support lymphoproliferation was assessed. Serum samples collected over a 1-yr period from 31 MAP-infected cows were compared with samples taken from 29 paratuberculosis-negative cows in the same age group. The cell proliferation assay is based on the fact that upon cell division, fluorescence intensity in response to a mitogenic stimulation is 50% lower in daughter cells than in the parental generation. Given that the lymphoproliferative capacity of some JD cows may be compromised (Waters et al., 1999), the capacity of their serum to sustain cell proliferation was evaluated. The serum from the JD cows was tested on peripheral blood mononucleated cells (PBMC) isolated from 3 different healthy cows (serum ELISA and FC negative), thus representing 3 biological replicates. Four serum samples from each cow (31 MAP-infected and 29 paratuberculosis-negative cows for a total of 60 animals) were individually (no pooling) tested for the capacity to support lymphoproliferation of PBMC from the healthy cows (3 independent assays). The percentage of cells proliferating in response to concanavalin A (**ConA**; Sigma-Aldrich, Oakville, ON, Canada) in the presence of serum from MAP-infected and paratuberculosis-negative cows was compared. Unless otherwise stated, all steps in the isolation were carried out at room temperature. Briefly, blood from the donor cows was collected from the jugular vein in 450-mL blood collection bags containing citrate dextrose. The buffy coat was collected from the interface following standard centrifugation in 50-mL sterile tubes at $1,200 \times g$ for 15 min, diluted 1:2 with room-temperature RPMI-1640 medium (Wisent, St-Bruno, QC, Canada), and layered

on Ficoll-Paque PLUS (GE Healthcare, Baie-d'Urfé, QC, Canada); centrifugation was then performed at $850 \times g$ for 35 min. The PBMC were washed with RPMI-1640 medium, resuspended in 4 mL of red blood cell lysing buffer (Sigma-Aldrich) for 2 min, and immediately diluted in RPMI-1640 medium; centrifugation was then performed at $100 \times g$ for 8 min. The cell count and viability were determined using the Countess Automated Cell Counter (Invitrogen, Burlington, ON, Canada). The cells were labeled with the Vybrant carboxyfluorescein diacetate succinimidyl ester (CFDA SE) Cell Tracer Kit (Molecular Probes, Burlington, ON, Canada) and diluted in Hanks' balanced salt solution (Wisent) at a concentration of $1 \mu\text{M}$ following incubation at 39°C for 20 min to ensure uniform marking of the cells. After centrifugation at $100 \times g$ for 8 min, excess CFDA SE was removed by incubating the cells in RPMI-1640 medium at room temperature for 20 min, and then the cells were pelleted at $100 \times g$ for 8 min. The cells were seeded at a density of 1×10^6 cells per well in 24-well plates containing antibiotic-free RPMI-1640 medium and 5% serum from negative or positive cows, supplemented with 200 mM L-glutamine (Wisent). Preliminary assays were performed to determine the concentration of cow serum and the concentration of ConA to be used. Cell proliferation was tested in the presence of 5 or 10% serum using a pool of heterologous (local herd) or JD cow serum and ConA at 0, 0.3, 0.5, 1.0, and 2.0 $\mu\text{g}/\text{mL}$ as described previously (Lessard et al., 2004; Doepel et al., 2006). The use of 10% cow serum showed reduced proliferation compared with 5% serum for both healthy and JD serum. The concentration of 5% cow serum was used as described elsewhere (Ster et al., 2012). Concentrations of 0.3 and 1 $\mu\text{g}/\text{mL}$ ConA were selected for the study. For the experiment (individual serum sample), 4 wells were used for testing each condition: no stimulation (control) and 0.3 or 1 $\mu\text{g}/\text{mL}$ ConA. Additional controls were performed for each condition (4 wells for the respective condition) using 5% fetal bovine serum (FBS) instead of cow serum. To avoid technical bias in the assays, each plate contained equal amounts of MAP-infected positive and negative cow sera. After a 3-d incubation period, the cells were pelleted in cytometry tubes, fixed in PBS-formaldehyde (4% vol/vol), and read in a 3-laser FACSCanto II flow cytometer (BD Biosciences). The intra- and interassay coefficients of variation (CV) were 3.2 and 8.6%, respectively.

Measurement of Plasma IFN- γ , IL-17, and IL-10 by ELISA

Bovine IFN- γ was measured using the Bovine IFN- γ ELISA Development Kit (Mabtech, Mariemont, OH) as

described by the manufacturer. The samples and standard curve were measured in duplicate. Interleukin-10 in plasma was measured using the Bovine Interleukin 10 ELISA Kit (Cusabio, Wuhan, Hubei Province, China) as recommended by the manufacturer. Interleukin-4 and IL-17 in plasma were measured using the ELISA Kit for Interleukin 4 and the ELISA Kit for Interleukin 17 (USCN Life Science, Houston, TX) as recommended by the manufacturer. All ELISA plates were read using a SpectraMax Plus384 microplate reader (Molecular Devices, Downingtown, PA). The intraassay CV for IFN- γ , IL-10, IL-4, and IL-17 were 5.3, 3.4, 6.5, and 7.0%, respectively.

Osteopontin Measurement in Plasma Samples

Plasma osteopontin concentration was measured using sandwich ELISA as previously described (Bissonnette et al., 2012). Briefly, 96-well ELISA plates (Greiner Bio-One North America, Monroe, NC) were coated with MAB193P (Maine Biotechnology Services, Portland, ME) diluted 1:1,000 in a coating buffer [0.05 M Na_2CO_3 buffer (pH 9.2)], sealed, and incubated at 4°C overnight. The plates were washed twice with PBS (pH 7.4) containing 0.05% Tween 20, incubated for 2 h with the blocking solution (PBS, pH 7.4, containing 1% fish skin gelatin), and finally washed 3 times before loading of the samples. The plates were incubated at room temperature for 1 h and then washed 3 times before the addition of the second antibody—a rabbit polyclonal antibody directed against bovine osteopontin (bOPN-121; Bissonnette et al., 2012)—diluted in the blocking solution. The plates were incubated for 2 h and then washed 6 times before the addition of the anti-rabbit IgG coupled to horseradish peroxidase NA934V (GE Healthcare). The plates were incubated for 1 h and underwent a final washing step; then, a solution of TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) was added to each well, and the plates were incubated for 10 min before stopping solution (2.0 M H_2SO_4) was added. The plates were read at 450 nm using a SpectraMax Plus384 microplate reader (Molecular Devices). Duplicates of the standard curve were included on every ELISA plate. The intra- and interassay CV were 2.5 and 6.3%, respectively.

Preparation of Bovine Monocyte-Derived Macrophages and Stimulation

For monocyte isolation, 700 mL of whole blood was collected from 8 healthy cows from the local MAP-free herd, and PBMC were collected as described above. Monocytes were isolated by adherence as described by Zhou et al. (2012) with minor modifications. Cells were

seeded at a density of 5×10^6 cells in 6-well, flat-bottomed plates in complete medium consisting of RPMI-1640 medium supplemented with 10% heat-inactivated bovine heterologous serum and $1 \times$ antibiotics and antifungals (Centrafarm, Etten-Leur, the Netherlands) at 39°C in a humidified atmosphere with 5% CO_2 . The cells were incubated for 2 h to allow monocytes to adhere, and then cells in suspension were removed. Following incubation for 18 h (d 1), the adhered cells were washed twice, and the medium was replaced with 2 mL of fresh antibiotic-containing medium to remove any nonadherent cells. The identity and purity of monocytes was confirmed by flow cytometry using an anti-CD14 Pacific Blue-labeled antibody as described by Taraktoglou et al. (2011), and CD14^+ cell purity greater than 99% was achieved (unpublished data). The cells were then incubated with RPMI-1640 medium containing 10% heat-inactivated FBS (Sigma-Aldrich) at 39°C in 5% CO_2 for 8 to 10 d. By d 10, 80 to 90% confluent monolayers displayed the characteristic macrophage morphology and confirmed purity greater than 98% using flow cytometry with an anti-CD68 antibody (unpublished data). The monocyte-derived macrophages were used for the in vitro challenge experiments. A multiplicity of infection of 10:1 (pathogen:macrophage) for the MAP pathogen (field strain 39382) was used to stimulate the cells, as described by Periasamy et al. (2013). Before the addition of MAP, macrophages were incubated for 24 h in antibiotic-free medium. Infection with viable MAP for time points 1, 6, 14, and 24 h was performed, and uninfected controls were harvested at 0 and 24 h. Assays were stopped by adding 1 mL of cell lysing buffer (RLT buffer; Qiagen, Toronto, ON, Canada) to each well.

RNA Extraction and Quantitative Real-Time PCR

The RNA from the macrophage infection assay was extracted and quantified by real-time PCR following reverse transcription (qRT-PCR) as previously described (Levesque-Sergerie et al., 2007), with minor modifications. All qRT-PCR reactions were performed in triplicate using 500 ng of total RNA. The cDNA was then diluted in molecular-grade H_2O and stored in multiple aliquots at -20°C for subsequent use. Primers were designed for each gene using the Primer Express 3 software package (Applied Biosystems, Life Technologies Corp., Burlington, ON, Canada) using the reference sequence from the RefSeq database (<http://www.ncbi.nlm.nih.gov/refseq/>) of the National Center for Biotechnology Information depository. Optimizations were performed for each gene by testing different concentrations of both forward and reverse primers, each ranging from 50 to 900 nM. Estimations of primer

efficiencies were analyzed using the standard curves made from a serial dilution of a pool of cDNA samples. Supplementary Table S1 (<http://dx.doi.org/10.3168/jds.2013-7059>) provides experimental information and PCR amplification efficiency for all genes. The qRT-PCR reactions (final volume of 20 μL) were performed on 96-well plates using Power SYBR Green PCR Master Mix (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The PCR thermal cycling conditions comprised an initial 20-s denaturation step at 95°C followed by 40 cycles at 95°C for 3 s and an annealing-elongation period at 60°C for 30 s. A dissociation step was included for all amplifications to confirm the presence of single discrete PCR products of the expected size; this was further confirmed by visualization of the amplification products on 2% agarose gels. The expression of 4 putative reference genes— β -actin (*ACTB*), cyclophilin A (*PPIA*), ubiquitously expressed transcript (*UXT*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*)—was determined for all samples. The *UXT* and *PPIA* genes were identified by BestKeeper (Pfaffl et al., 2004) as the most stable genes. Pairwise analysis of expression ratios using the geNorm software (Vandesompele et al., 2002) was used to confirm *UXT* and *PPIA* as internal controls. Their geometrical means were used for normalization of longitudinal gene expression profiling as described previously (Bionaz and Looor, 2007). The qRT-PCR results were analyzed according to the comparative cycle threshold (C_T) method, given by the arithmetic formula $2^{-(\Delta\Delta C_T)}$ (Livak and Schmittgen, 2001).

Statistical Analysis

Differences in lymphoproliferation were analyzed using one-way ANOVA and Tukey's multiple comparison tests. Differences in plasmatic cytokine secretion were determined on transformed data using ANOVA and Tukey's multiple comparison tests for IL-17, IFN- γ , and osteopontin. Plasma concentrations of IL-10 and IL-4 were analyzed using ANOVA and Tukey's multiple comparison tests. The qRT-PCR data were analyzed using average fold-change analysis in combination with Student's *t*-test.

RESULTS AND DISCUSSION

Currently, it is difficult to detect cows with subclinical MAP infections. The difficulty stems from the lack of sensitivity of serum ELISA during the early period. During the more advanced and clinical stages of the disease, animals develop high levels of antibody

response, which explain why serological detection performs better in older animals (Nielsen et al., 2002; Norton et al., 2010). To explain that change, it was first suggested that a T_{H1} (cell-mediated; IFN- γ) and T_{H2} (humoral; specific antibody) imbalance occurs over time (Whittington et al., 2012 and references therein). However, studies on the progression of JD have indicated that the immune responses to MAP infection are more complex than previously thought. In cattle that progress to clinical disease, the cell population diversity changes, leading to an anergic state (Waters et al., 1999; Stabel, 2000, 2006). Recently, evidence for a population of regulatory T cells has also begun to accumulate (Coussens et al., 2012). This assumption derives from cross-species studies, characterization of cell population, and ex vivo challenges using commercial serum (e.g., FBS) most of the time. In the present study, we measured lymphoproliferative activity and obtained evidence that serum from MAP-infected cows dampened cell proliferation. The PBMC incubated in the presence of serum from infected cows and stimulated with ConA showed lower proliferation (Figure 1; $P = 0.001$) compared with the proliferation measured in the presence of serum from paratuberculosis-negative ($-/-$) cows. The proliferative capacity of the PBMC was significantly reduced in the presence of 0.3 $\mu\text{g/mL}$ ConA, a difference that was lost using a concentration that maximized proliferation (1.0 $\mu\text{g/mL}$ and above). Serum samples from 29 healthy and 31 diseased cows were tested individually (not pooled). Proliferation was about 63% in the presence of serum from healthy cows and decreased to 46% in the presence of serum from subclinical cows. This observation was confirmed in 3 distinct assays using PBMC isolated from 3 different noninfected cows (biological replicates). We previously observed that autologous serum was less prone to support cell proliferation than was FBS (Lessard et al., 2004; Doepel et al., 2006). In the present study, lower cell proliferation in the presence of cow serum was also observed (63% compared with 83% with FBS), and the reduction was even greater with the serum of MAP-infected cows (46%). These observations suggest that the lymphoproliferative capacity of JD cows may be compromised at the whole-animal level. This lower capacity may be explained either by the presence of inhibitory factors or by impoverishment of the serum content. As reported previously, cows experiencing metabolic stress have reduced lymphoproliferative capacity (McCarthy et al., 2010; Ster et al., 2012). The high metabolic demands resulting from the challenge to the gut system might explain the serum impoverishment in these 31 subclinical cows. Another possible explanation is that the inhibition is associated with the presence of host or mycobacterial factors. Some authors have

shown that serum from *M. bovis* (Lamont et al., 2014) and MAP-infected cows (You et al., 2012) contains specific host and mycobacterial proteins. Some of these biomarkers have biological activities and their potential influence cannot be excluded. The mechanisms leading to the development of the immune dysregulation observed in clinical cows with chronic gut inflammation are not yet understood. Our results suggest that serum from bovine paratuberculosis cows is associated with a reduced capacity to support lymphoproliferation.

It has been suggested that an immunological switch occurs as the disease progresses (Stabel, 2000). However, the status (ELISA-positive or negative) of the subclinical cows did not influence cell proliferation. The response of the ELISA-negative MAP-shedding cows yielded proliferation levels similar to that of the serum from the ELISA-positive cows ($P = 0.20$). In other words, the serum from both of the MAP-shedding

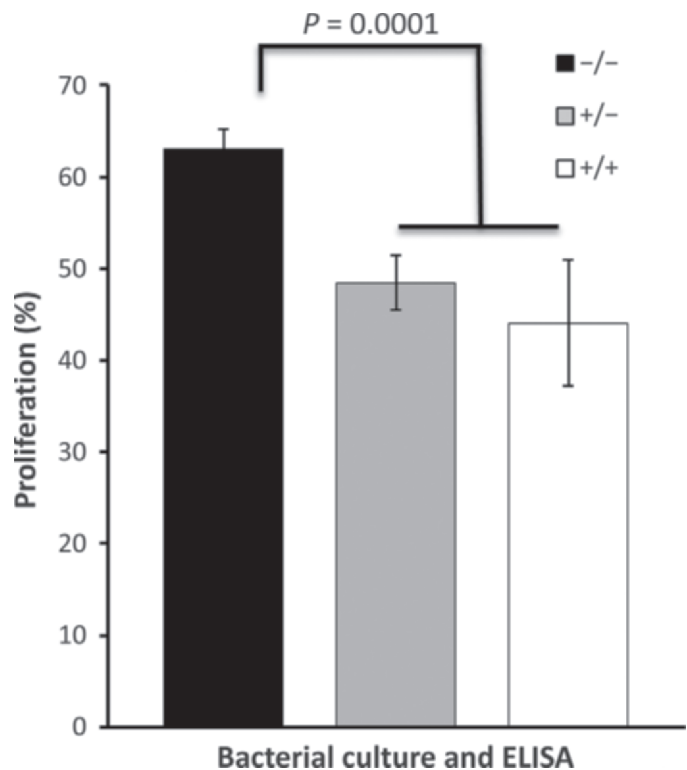


Figure 1. Lymphoproliferation in presence of serum from subclinical cows excreting *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in their feces and classified ELISA positive (+/+) or negative (+/-) compared with the serum from healthy cows (-/-). The peripheral blood mononuclear cells isolated from 3 healthy cows were used to measure the capacity of the serum from cows infected with bovine paratuberculosis to support the proliferative response to concanavalin A (ConA). Data are shown as means $\pm$ SEM for negative ($n = 29$), single-positive (+/-; $n = 22$, meaning positive from only the bacterial culture of fecal samples), or double-positive (+/+, meaning positive from both bacterial culture and serum ELISA; $n = 9$). Statistical analysis was performed using ANOVA with Tukey's corrections.

groups (infectious and subclinically infected cows) produced a similar limiting effect on lymphocyte proliferation. According to the T_{H1}/T_{H2} shift hypothesis, cows become ELISA-positive as the switch occurs. Given that IFN- γ and IL-4 are the T_{H1} - and T_{H2} -associated cytokines, respectively, plasma levels of IL-4 and IFN- γ (Figures 2C and B, respectively) were measured. The levels of IL-4 in the MAP-infected cows were similar to those in the uninfected cows (Figure 2C; $P > 0.07$), but IFN- γ blood concentrations were more elevated in both positive groups than in the negative group (Figure 2B; $P = 0.01$). These results do not fit the T_{H1}/T_{H2} shift paradigm. They do, however, agree with the increased IFN- γ levels observed in the shedding stage of subclinical JD (Sweeney et al., 1998). Many studies have reported an increase in IFN- γ expression in the tissue and PBMC of subclinical cows (Sweeney et al., 1998; Coussens et al., 2004) and JD-positive cows (Shu et al., 2011), but to our knowledge, this is the first time that a higher IFN- γ concentration has been reported in the plasma of MAP-infected cows. Interferon- γ helps to activate macrophages for pathogen degradation. However, MAP dampens the responsiveness of its host to IFN- γ stimulation by increasing IL-10 production locally. In infected gut tissue, it results in lower IFN- γ expression by $CD4^+$ T cells (Coussens, 2001), which might contribute to its tolerance. Interestingly, the infected cows with serologically negative results had a reduced IL-4 blood level (Figure 2C; $P = 0.03$) compared with the ELISA-positive cows [double-positive (+/+) group]. B cells are mature in their capacity to switch to immunoglobulin-producing cells if they are given exogenous IL-4. Defective immunoglobulin production has been attributed to impaired T_H cell functions or suppressor T cell activity (Coffman, 2006). The reduced IL-4 level of these serum ELISA-negative MAP-shedding cows suggests that these cows might experience a deficient T_{H2} function, which in turn explains the lack of effective adaptive immunity, as observed by the absence of MAP-specific antibodies.

On the basis of studies in humans and mice infected with *M. tuberculosis*, it was suggested that dysregulation of cytokine signaling leads to induction of T_{reg} cells (Kursar et al., 2007; Sharma et al., 2009). It is postulated that T_{reg} cells play a similar role in bovine paratuberculosis (de Almeida et al., 2008). The reduced proliferative capacity of MAP-positive serum could lead to the hypothesis that such serum contains antiinflammatory cytokines. In the present study, however, the plasma concentration of IL-10 did not differ between the groups (Figure 2A; $P = 0.40$). Although a local effect cannot be ruled out (Weiss et al., 2005), the present observation suggests that IL-10 may not be implicated in the reduced systemic proliferative capac-

ity observed with the serum from the bovine paratuberculosis-infected cows. More recent findings regarding the response of PBMC to live MAP stimulation suggest that T_{H1} and T_{H17} cytokines, rather than T_{reg} -associated factors, are associated with bovine paratuberculosis (Park et al., 2011). The implication of T_{H17} lineage cells in the pathogenesis of Crohn's disease has been shown (Brand, 2009). Given the resemblance between JD and Crohn's disease, plasma IL-17 levels were quantified in the present study, and higher levels were found for the double-positive group (Figure 2D; $P = 0.001$). Similar to the results obtained for the IL-4 cytokine, the plasma IL-17 level in the single-positive (+/-) cows was also lower (Figure 2D; $P = 0.02$). This finding suggests that these ELISA-negative cows, which did not exhibit a high humoral immune response, were silent shedders at a less advanced stage but were already showing reduced serum-associated lymphoproliferative capacity. How do we reconcile the paradoxical state observed as the disease progresses? The intestinal infection that occurs in these shedding cows induces suboptimal gut performance and would have the consequence of impoverishing the serum, thus producing a systemic effect on lymphocyte proliferation, which is a real advantage for this slow-growing pathogen. The apparent contradictory antilymphoproliferative and proinflammatory data observed in the present study seem to support 2 distinct inflammatory states. Interestingly, similar results were observed by Verschoor et al. (2010), whose data from the microarray analysis of PBMC isolated from infected cows suggest a mix of pro- and antiinflammatory phenotypes. A concomitant effect of cytokines on T cell differentiation might occur during the progressive evolution of chronic gut inflammation in paratuberculosis-infected cows. Indeed, the presence of proinflammatory cytokines favoring the differentiation of suppressive T_{reg} subsets also supports T_{H1}/T_{H17} cell differentiation (Brand, 2009). The paradox of having both pro- and antiinflammatory conditions has been described several times in the development of autoimmune diseases (Su et al., 2012). The presence of T_{H17} inflammatory signals could highlight a problem with T_{reg} in terms of their role in suppressing the inflammation. The osteopontin levels in the present study further support the idea of the chronic inflammatory state of the disease. Osteopontin is suspected to play a role in the secretion (Murugaiyan et al., 2008), regulation (Shinohara et al., 2008), and differentiation (Chen et al., 2010) of naive $CD4$ T cells with T_{H17} effector activity. In the present study, a higher osteopontin concentration was measured in the MAP-infected cows (Figure 2E). Compared with the level in the noninfected cows, osteopontin was slightly but significantly more elevated in the blood from the single-positive

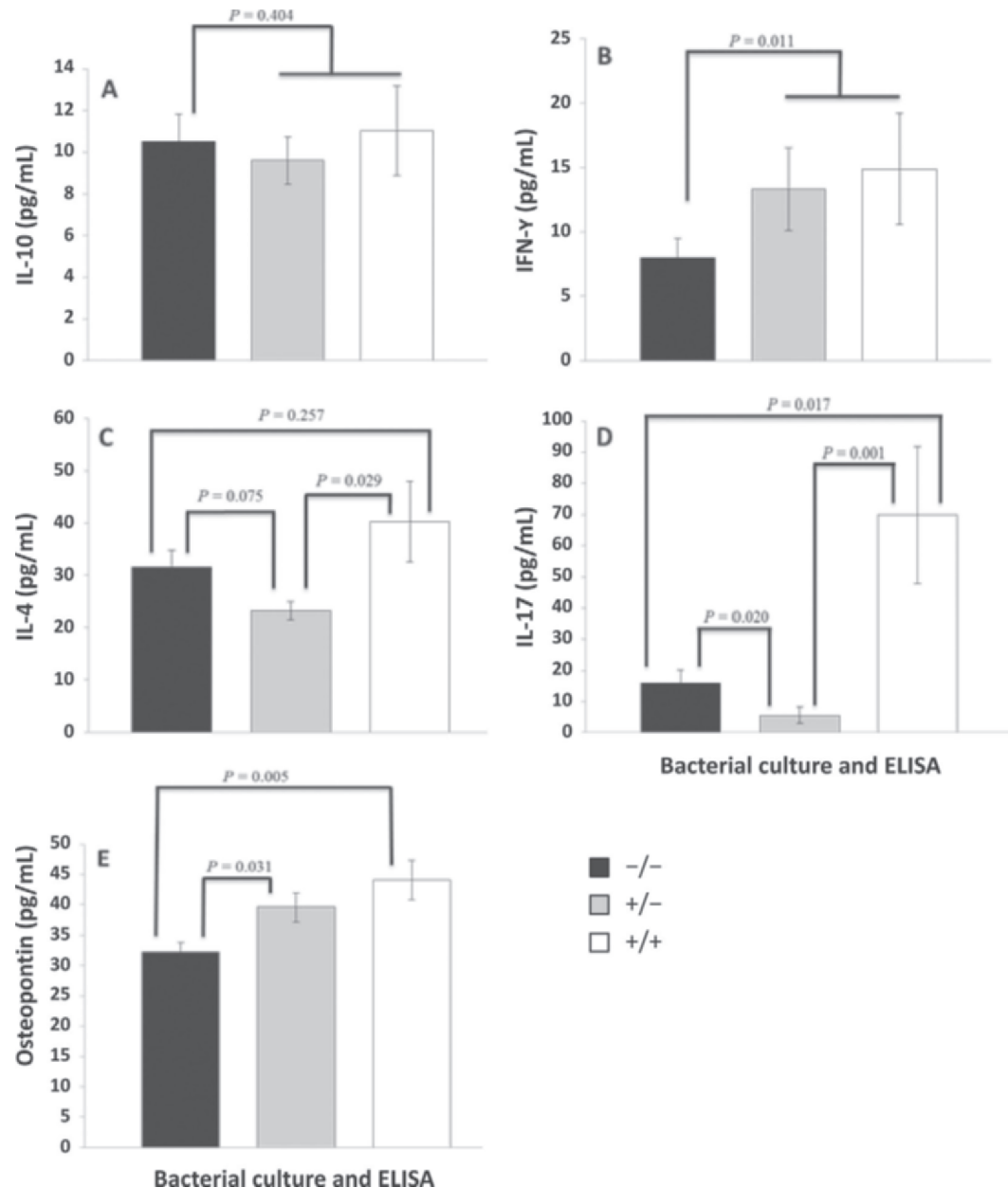


Figure 2. Plasma concentrations of cytokines IL-10 (A), IFN- γ (B), IL-4 (C), IL-17 (D) and osteopontin (E). The cows were classified according to their status as negative (-/-; $n = 39$), single-positive (+/-; $n = 32$, meaning positive from only the bacterial culture of fecal samples), or double-positive (+/+, meaning positive from both bacterial culture and serum ELISA; $n = 9$). Data are shown as means $\pm$ SEM. Statistical analysis was performed using ANOVA with Tukey's multiple corrections.

group of cows ($P = 0.03$) and nearly 25% higher in the blood from the double-positive group ($P = 0.004$). The osteopontin level in the noninfected cows was similar to the plasma level measured in a previous study in 15 cows sampled weekly during a full lactation period (unpublished data). The blood level of the osteopontin cytokine is thus highly stable. Interestingly, given that osteopontin is a marker for inflammatory bowel disease (Neuman, 2012) and a genetic factor predisposing to

susceptibility to Crohn's disease (Glas et al., 2011), the higher osteopontin level in these MAP-infected cows is associated with the proinflammatory condition prevailing in bovine paratuberculosis.

In support of this new T_{H1}/T_{H17} paradigm, the gene expression of MAP-infected macrophages was analyzed to study the cytokine profile, which is considered to support naïve T cell differentiation into T cell subsets. The analysis confirms that the secretion of cytokines

may play a pivotal role in driving the differentiation of naïve CD4 T cells into the type of subsets with T_{H17} effector activity. The levels of *IL-1 β* , *IL-6*, and *IL-23* transcripts were quantified by qRT-PCR, and upregulation was observed as early as 1 h postinfection (Figure 3; $P < 0.05$). Interestingly, these cytokines are implicated in the differentiation or maintenance of human or mouse T_{H17} cells (Bettelli et al., 2006; Manel et al., 2008; Jansson et al., 2012), and the cytokines' high levels persisted for longer infection periods (Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2013-7059>). The *TGF- β* gene was slightly (~1.5-fold) induced at 14 h and for a longer infection period (Figure 3; $P < 0.05$). Although the cooperation of *TGF- β* with *IL-6* supports naïve T-cell differentiation into T_{H17} effector cells, *IL-23* is associated with the clonal expansion of these cells. Indeed, T_{H17} cells are differentiated by a combination of the cytokines *TGF- β* , *IL-6*, and *IL-1 β* , which induce ROR γ t, a transcription factor required for the generation of these cells (Mangan et al., 2006). Whereas *TGF- β* and *IL-6* can induce T_{H17} cells, exposure to another cytokine, *IL-23*, is crucial for the clonal expansion and stabilization of this cell type and to support their ability to induce autoimmune tissue inflammation. Additionally, the proinflammatory tumor necrosis factor- α and *IL-8* genes were abundantly expressed in bovine macrophages and increased, respectively, by nearly 104 ± 41 times (at 6 h; $P = 0.02$) and 15 ± 3 times (14–24 h; $P < 0.001$). Antiinflammatory *IL-10* expression was also induced throughout the infection period ($P < 0.01$) but to a lesser extent. As the infection progressed, *IL-10* was less abundant and decreased to 2-fold at 24 h. This transient increase in *IL-10* may be induced by the pathogen to promote the establishment of the infection (Weiss et al., 2005). Indeed, the capacity of *IL-10* to impair phagosome maturation and delay antigen processing (Bobadilla et al., 2013) could be used by the pathogen to prevent its recognition and degradation by the host's cells. Although tumor necrosis factor- α cooperates with *IL-17* in the proinflammatory processes induced in many autoimmune diseases (Ouyang et al., 2008), these proinflammatory cytokines may contribute to the recruitment of neutrophils and other immune cells. Interleukin-12 is involved in T_{H1} differentiation and is inhibitory toward a T_{H17} response. In the current study, *IL-12* expression increased but was not significant (Figure 3), thus providing evidence to support the interpretation of a signaling bias toward T_{H17} differentiation. The in vitro macrophage infection assays with live MAP provide a better understanding of the initial processes in place. These results support the hypothesis that macrophages infected with MAP lead to a global dominance of a T_{H17} -polarizing environment.

Results from this project may contribute to a better understanding of the chronic inflammation that develops during the progression of bovine paratuberculosis. The different cytokine profiles observed between the experimental groups suggest that a thorough characterization of the immune profile associated with bovine paratuberculosis is worth consideration in studying the progression of JD. In addition, the factors identified in the serum of paratuberculosis-infected cows as impairing the proliferative capacity of the lymphocyte cells could turn out to be useful markers for diagnosis. Taken together, these findings support the idea that further investigations are needed to better understand this complex disease.

CONCLUSIONS

Bovine paratuberculosis results in dysregulation of the gut immunity that might induce serum impoverishment in these infected dairy cows, which in turn may impair the capacity to maintain a protective immune response. Another possible explanation is that the inhibition is associated with the presence of host or mycobacterial factors. The reduced lymphoproliferative capacity was not associated with *IL-10* as plasma levels of the MAP-infected cows were similar to those of the noninfected cows. However, both IFN- γ and osteopontin levels were higher in the MAP-positive cows, including those from the ELISA-positive groups. Interestingly, the MAP-shedding cows with positive ELISA also had higher blood levels of *IL-17*, evidence of a systemic proinflammatory T_{H17} state. Gene expression profiling of in vitro bovine macrophage infection with live MAP supports the hypothesis of a T_{H17} lineage commitment with upregulation of most of the cytokines required for the differentiation of naïve T lymphocytes into a T_{H17} subset. These results do not fit into the classical T_{H1} / T_{H2} hypothesis. Rather, existing data from the present study and others point to sequential windows toward a proinflammatory T_{H17} shift for cows with subclinical bovine paratuberculosis, similar to the situation in other chronic intestinal bowel diseases.

ACKNOWLEDGMENTS

The authors thank G. Côté, E. Doré, and S. Buczinski (all from Faculté de Médecine de l'Université de Montréal, Saint-Hyacinthe, QC, Canada), for their assistance for coordinating the herd visits, Catherine Thibault and Karine Deschêne (both from Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada) for providing technical assistance with sampling and cell culture and Karoline Lauzon and Laurine Gil (both

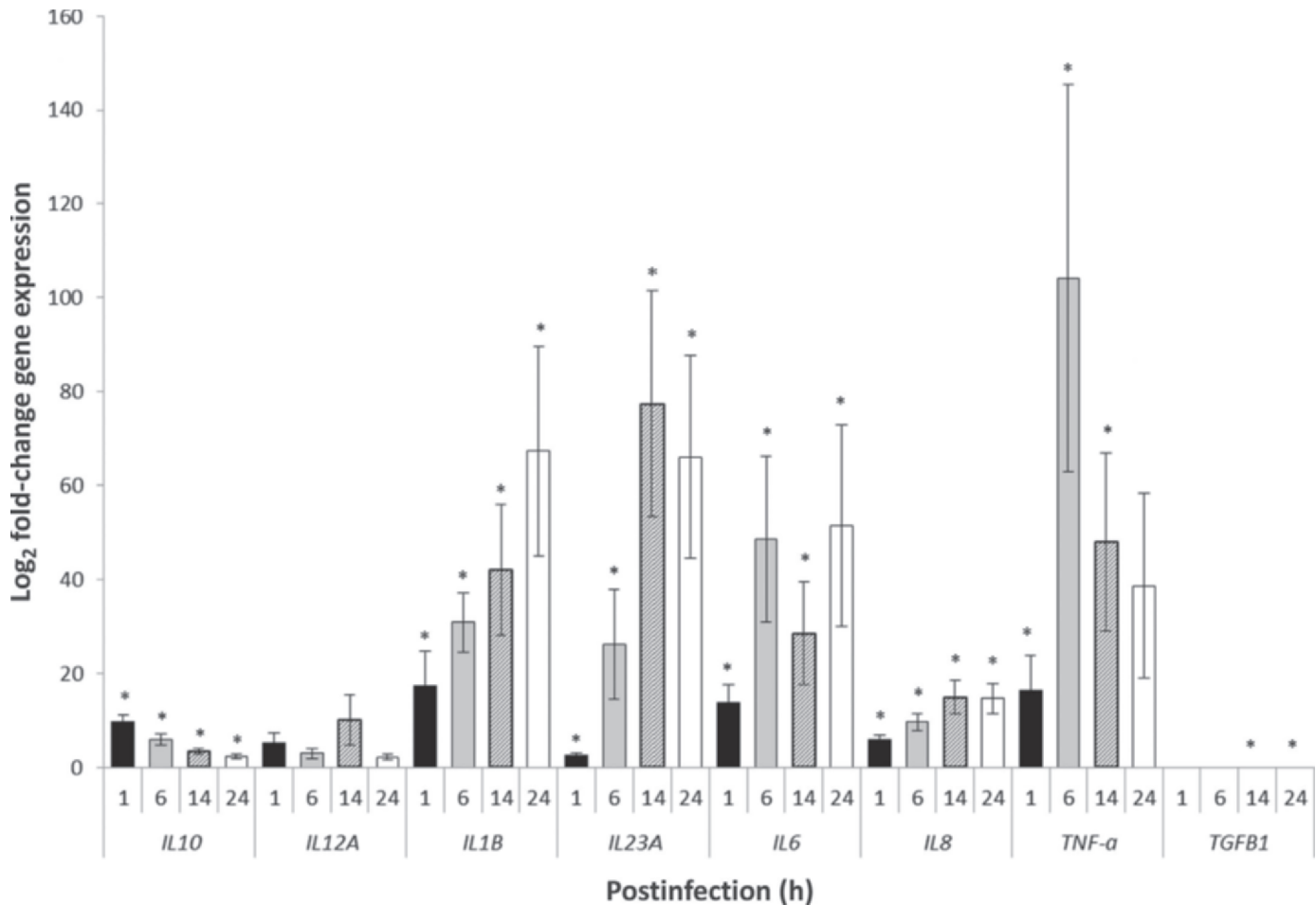


Figure 3. Transcription of cytokines in bovine macrophages infected with live *Mycobacterium avium* ssp. *paratuberculosis*. Total RNA was extracted from macrophages after 1, 6, 14, and 24 h of infection, and the gene expression of cytokines was measured by quantitative real-time PCR. The relative gene expression (fold increase) was calculated using 2 housekeeping genes (*PPIA* and *UXT*) and the uninfected macrophages (negative control) as normalized by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Data are shown as the means of 3 independent assays. The error bars indicate standard error; * indicates a significant difference compared with cytokine transcription in the negative control ($P < 0.05$).

from Agriculture and Agri-Food Canada) for performing ELISA assays. This research was supported by Agriculture and Agri-Food Canada, Novalait Inc. (Québec, QC, Canada), Fonds québécois de la recherche sur la nature et des technologies, and the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (Québec, QC, Canada; grant no. RBPI-2037).

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