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ScienceDirect
Veterinary Immunology and Immunopathology 116 (2007) 131–144
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Relationship between *Mycobacterium avium* subspecies *paratuberculosis*, IL-1α, and TRAF1 in primary bovine monocyte-derived macrophages

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Received 18 October 2006; received in revised form 5 January 2007; accepted 16 January 2007

Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a facultative intracellular pathogen that resides in host macrophage cells. Presently, little is known about how MAP is able to subvert the normal bacteriocidal functions of infected macrophages. Previously, we reported that ileal tissues from MAP infected cattle contained high levels of interleukin-1 alpha (IL-1α) and tumor necrosis factor receptor-associated factor 1 (TRAF1), relative to ileal tissues from uninfected cattle. High-level expression of these two proteins could have profound effects on macrophage function, intracellular signaling, and apoptosis. We now demonstrate that high levels of TRAF1 protein are located primarily within macrophages infiltrating areas of MAP infection. We have also utilized cultured bovine monocyte-derived macrophage cells (MDM) either infected with live MAP or stimulated with recombinant IL-1α (rIL-1α) to determine if there is a relationship between IL-1α and TRAF1 expression. These studies have identified a dose dependent increase in TRAF1 protein levels in bovine MDM in response to infection with live MAP or following treatment with rIL-1α. Sustained TRAF1 protein expression was dependent upon interaction of rIL-1α with it's receptor and rIL-1β was also able to enhance TRAF1 gene expression. Our results suggest that MAP may use the IL-1-TRAF1 system to enhance TRAF1 protein expression in infected bovine MDM. These novel results provide evidence for a new avenue of research on the effect of MAP and other intracellular pathogens on macrophage signaling and apoptosis.

Keywords: Johne's disease; CD40; TNFR1; TNFR2; Macrophage

1. Introduction

Johne's disease is a chronic infection of ruminants resulting in localized inflammation and granulomatous lesion formation within the gut. The causative agent of Johne's disease is the facultative intracellular bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Buergelt et al., 1978; Harris and Barletta, 2001). Johne's disease is found worldwide, and the average economic loss within the dairy industry due to Johne's disease has been calculated to equal $227.00 for each individual cow in an infected herd per year (NAHMS, 1997). There is a still controversial, but developing link between MAP and human Crohn's

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doi:10.1016/j.vetimm.2007.01.005

disease with MAP organisms being isolated from intestinal tissue, blood, and breast milk from a subset of Crohn's disease patients (Naser et al., 2000, 2004). In some cases, anti-mycobacterial therapy has a direct clinical benefit to Crohn's patients (Hermon-Taylor, 2002).

Initial infection of cattle with MAP typically occurs before six months of age. The route of infection may follow one of several pathways, including fecal–oral transfer, in utero infection, or by ingestion of colostrum from an infected dam (Seitz et al., 1989; Streeter et al., 1995; Sweeney, 1996). Once inside the host it is suspected that MAP crosses epithelial linings of the gut through membranous epithelial cells (M cells) where the pathogen is phagocytosed by local macrophage cells (review, Stabel, 2000). Upon phagocytosis of MAP by näive macrophages, normal processing through the endocytic pathway may be arrested, with MAP containing phagosomes failing to acquire significant amounts of LAMP-1 and to fuse with lysosomes, thus allowing MAP to survive and proliferate inside macrophages (Bannantine and Stabel, 2002; Hostetter et al., 2003; Valheim et al., 2004; Zapata et al., 2000; Zurbrick and Czuprynski, 1987).

Disease pathology characteristic of ruminants infected with MAP is typically focused near the ileocecal valve, ileum, jejunum and adjacent areas. In cattle, outward clinical signs of infection and bacterial shedding are usually not evident until 2–5 years post-infection (Begara-McGorum et al., 1998; Buergelt et al., 1978). During the subclinical non-shedding phase, MAP continues to proliferate within macrophages of the gut. Animals usually appear healthy and exhibit no outward signs of Johne's disease when they first begin shedding MAP in their feces, thus perpetuating the infection within a herd. It is during this late subclinical shedding phase that animals may first be definitively diagnosed by fecal culture. Lowered milk production, weight loss, and persistent diarrhea mark the onset of the final clinical stage of Johne's disease.

Common pathology of clinical Johne's disease within cattle is diffuse granulomatous lesions with mucosal thickening and large numbers of acid-fast staining (infected) macrophages (Buergelt et al., 1978; Corpa et al., 2000; Lee et al., 2001). Failure of macrophages to properly clear MAP, combined with an influx of macrophages and lymphocytes results in a Thl-like proinflammatory condition at the site of infection (Valheim et al., 2004). It is presently not known if the dramatic increase in numbers of macrophages typically associated with lesions in Johne's disease is due to prolonged survival of infected cells, to enhanced migration of monocytes to sites of infection, or to both. Despite expression of IL-8 and other chemokines, there is a distinct lack of neutrophils in lesions of cattle with advanced Johne's disease.

Previous studies conducted by our group have reported enhanced expression of the proinflammatory cytokine IL-1α within ileal tissues from Johne's positive cattle (Aho et al., 2003). IL-1α, along with tumor necrosis factor alpha (TNFα), has been linked to promotion of granuloma formation and maintenance in mycobacterial infections (Juffermans et al., 1998, 2000). Presence of IL-1α is consistent with large numbers of infiltrating macrophages present in granulomatous lesions characteristic of clinical Johne's disease. High levels of proinflammatory IL-1α at the site of infection are also in agreement with observed Thl-like inflammatory activity in ileal tissues of MAP infected cattle.

Our group has also previously reported enhanced TRAF1 gene expression and protein levels in ileal tissues from Johne's disease positive cattle relative to uninfected control cattle (Aho et al., 2003). TRAF proteins function as adaptor or scaffolding peptides, helping to transfer signals from a variety of cell surface receptors, but they do not possess intrinsic enzymatic properties (Arron et al., 2002). TRAF1 is a strongly anti-apoptotic protein involved in signaling from TNFα receptor superfamily members (Durkop et al., 2003; Pryhuber et al., 2000; Speiser et al., 1997; Wang et al., 1998). TRAF1 in heterotrimeric complexes with TRAF2 has been shown to interact with the cytosolic domains of CD40, CD30, tumor necrosis factor receptor-1 (TNFR1), and tumor necrosis factor receptor-2 (TNFR2) (Durkop et al., 2003; Pryhuber et al., 2000; Pullen et al., 1998; Speiser et al., 1997; Wang et al., 1998). The outcome of these interactions, enhanced gene expression, proliferation, or apoptosis, is in large part dependent upon the ratio of TRAF1 and TRAF2 in complexes, with excess TRAF1 tending to promote cell survival. Lymphocytes, macrophages, and dendritic cells, are known to produce TRAF1 (Bradley and Pober, 2001).

Although intriguing, previous observations did not explain the potential relationship between MAP infection, IL-1α expression, and TRAF1 expression. In addition, previous studies did not provide evidence for the cell types involved in high-level expression of IL-1α and TRAF1 protein in lesions of MAP infected cattle. In this report, we applied immunohistochemical techniques to determine specific cell-types responsible for increased production of IL-1α and TRAF1 proteins within ileal tissues from MAP infected cattle. Enhanced TRAF1 protein production within ileal lesion from MAP infected cattle was specifically localized to

macrophages. Furthermore, IL-1α proteins were present at higher levels in infected tissue relative to control (Aho et al., 2003), however the diffuse staining pattern suggested that most IL-1α in infected lesions was secreted and thus expression could not be assigned to any specific cell type.

Based upon initial immunohistochemical evidence, we next investigated TRAF1 protein levels in cultured bovine monocyte-derived macrophages (MDM) challenged with live MAP or treated with rIL-1α in vitro. Results presented in this report indicate that cultured bovine MDM constitutively express small amounts of IL-1α and exhibit enhanced expression of TRAF1 mRNA and protein in a dose dependant manner when challenged with live MAP, with rIL-1α, and with rIL-1β. Perturbation of the IL-1:TRAF1 system by MAP provides evidence for a new avenue of research on the effect of MAP and other intracellular pathogens on macrophage signaling, function, and activation.

2. Materials and methods

2.1. Bacterial cultures

M. avium subspecies *paratuberculosis* (MAP) was obtained from the American Type Culture Collection (ATCC #19698) and grown at 37 °C in Middlebrooks 7H9 media with 10% Middlebrooks OADC enrichment and Mycobactin J (Allied Monitor, Lexana, KS) at 2 mg/l for 12–16 weeks. MAP were serially diluted and counted on a bacterial hemocytometer. Prior to infection of macrophage cells, bacterial suspensions were vigorously vortexed to disperse clumps and diluted in phosphate buffered saline (PBS).

2.2. Experimental animals

Uninfected control cattle used in this study as a source of tissues ($n = 2$) and monocyte-derived macrophages ($n = 5$) were all of the Holstein breed and housed at the Michigan State University Dairy farm. Control cattle ranged in age from 12 to 48 months. The immune status of control cattle with regard to infection with MAP had been monitored by serum enzyme-linked immunosorbent assay (ELISA) and periodic interferon gamma (IFNγ) testing (Bovigam, Biocor Animal Health Inc., Omaha, NE) prior to initiation of experiments. Fecal culture testing by a U.S. Department of Agriculture-approved testing laboratory (Michigan State University Diagnostic Center for Population and Animal Health, East Lansing, MI.) was conducted to confirm negative infection status for control cattle. Control cattle were serum ELISA negative, IFNγ negative, and fecal culture negative. Clinical infected cattle ($n = 3$) used as a source of ileal tissues were from a commercial herd in Michigan. Clinical cattle were serum ELISA and fecal culture positive (5–100 CFU/g of feces), had variable response to IFNγ testing, and exhibited outward signs of infection including intermittent diarrhea, and moderate weight loss.

2.3. Tissue collection

Ileal tissues were collected at the time of necropsy (infected) or slaughter (controls). All cattle were macroscopically examined for pathological signs of Johne's disease. All MAP infected cattle exhibited thickening of the ileal mucosa, convolution of the interior of the ileum, and enlargement of the adjacent draining lymph nodes. All control cattle exhibited healthy ileum and mesenteric lymph nodes, with no visible pathological signs of Johne's disease. Three individual sections were harvested from each animal from regions immediately adjacent (within 20 cm) to the ileal–cecal junction. Tissue sections were immediately placed into 50 ml conical vials containing Safefix (Biochemical Science Inc., Swedesboro, NJ) and stored at −80 °C until slides were prepared.

2.4. Slide preparation and immunohistochemical staining

Following fixation in Safefix (Biochemical Science Inc.) for histopathologic examination, sections of ileum were trimmed, embedded in paraffin, sectioned at 5 μm and processed for immunohistochemistry. Formalin fixed, paraffin embedded sections of ileum from cows that had microscopic lesions consistent with Johne's disease and been previously tested positive for MAP infection served as positive tissue controls and sections of ileum from histologically unremarkable cows were used as negative controls. In addition, separate sections from all tissues were treated with homologous non-immune sera as secondary antibody controls.

Slides were cut, and subsequently baked in a drying oven at 60 °C for 30 min. The slides were then bar-code labeled and placed in the Benchmark IHC system (Ventana, Tucson, AZ). Deparaffinization and heat induced epitope retrieval was accomplished on the Benchmark. Antigen retrieval was performed using retrieval solution CC2 (Ventana) with an 8 min heating and an 8 min cooling cycle. A rabbit-polyclonal anti-TRAF1 antibody (sc-7186, Santa Cruz Biotechnologies, Santa Cruz, CA) was used as the primary antibody

at a dilution of 1:10 for 32 min. Binding of TRAF1 primary antibody was detected using an alkaline phosphatase-conjugated secondary antibody and an enhanced alkaline phosphatase red kit, as well as bulk buffers specifically designed for use on the Benchmark IHC System. The slides were counterstained using Ventana hematoxylin (Ventana) and bluing for 2 min each, then dehydrated, cleared and mounted.

2.5. Preparation of bovine monocyte-derived macrophages

For isolation of primary bovine monocyte-derived macrophages (MDM), blood was collected from the tail vein of healthy donor cattle using 8 ml vacutainers (BD Inc., Franklin Lakes, NJ) containing 1.5 ml acid citrate dextrose as anticoagulant. Blood was centrifuged for 20 min at 600 × *g* and 4 °C and plasma was removed. Buffy coat cells were transferred to 50 ml conical tubes containing 10 ml cold sterile Percoll (1.084 g/cm^3, Sigma, St. Louis, MO) layered under 30 ml of cold sterile PBS and centrifuged for 40 min at 400 × *g* at room temperature. Mononuclear cells were collected from the PBS–Percoll interface and washed one time with cold PBS. For preparation of bovine MDM, isolated mononuclear cells were adjusted to 6.8×10^6 cells/ml, plated in 6-well culture dishes, and incubated for 2 h in 5% CO_2 in RPMI 1640 tissue culture medium (Gibco Inc., Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco Inc., Grand Island, NY) at 39 °C to allow monocytes to adhere.

After 2 h incubation, nonadherent cells were removed by washing three times with warm (39 °C) PBS. Adherent monocyte cells were allowed to differentiate in culture for 7 days in 5% CO_2 in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum at 39 °C. Following differentiation, MDM cells were checked for morphology using a light microscope. The final yield of MDM cells was approximately 2×10^6 cells/well. Prior to various treatments (MAP, rIL-1α, rIL-1β, rIL-1RA), MDM cells were washed 3 times with warm PBS and all treatments were conducted in RPMI 1640 tissue culture medium without phenol red supplemented with 10% fetal bovine serum.

2.6. Infection of bovine MDM with MAP

Bovine MDM from 5 healthy control cattle were cultured in RPMI 1640 tissue culture medium without phenol red supplemented with 10% fetal bovine serum and infected with MAP at different multiplicities of infection (MOIs) of 1, 5, and 10:1 (1, 5, and 10 bacilli/macrophage) for 2 h. Cells were washed two to three times with warm PBS to remove uningested bacteria and RPMI medium containing 10% fetal bovine serum was replaced. For protein assays, infections were allowed to progress for 24 h, or the indicated times in time course studies. For RNA isolation, cells were infected for 6 h or the indicated times in time course studies. In each case, cells collected immediately after washing were designated as "0" time. For protein assays, cells were lysed in radioimmune precipitation assay buffer (RIPA; 1% Triton-X, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.02 M Tris–HCl). Lysates were collected and stored at −80 °C until use in Western blotting. In cases where recombinant IL-1 receptor antagonist (rIL-1RA) was used, 100 pg/ml of rIL-1RA (R&D systems, Minneapolis, MN) was added to MDM cell cultures 30 min prior to infection with MAP. RNA was extracted as described under quantitative real time RT-PCR and stored at −80 °C until use.

2.7. Flow cytometric analysis of MAP infection

To determine the efficiency with which MAP was able to infect MDM cells under our culture conditions, approximately 1×10^6 MDM cells were seeded in each well of 6-well culture plates and allowed to settle overnight at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. Cells were then infected with a green fluorescent protein (GFP) expressing MAP strain (kindly provided by Dr. Raul Barletta, University of Nebraska, Lincoln, Nebraska) at various MOIs ranging from 1:1 (MAP:Cells) up to 10:1. After 2 h, cells were rinsed three times with warm PBS and harvested by scraping into 1 ml of PBS. Cells were analyzed for uptake of GFP expressing MAP using flow cytometry on a Becton Dickinson FACSCalibur. The *y*-axis was set to display side scatter (SSC-H) and the *x*-axis was set to display fluorescence in the green range (FL-1-H). Uninfected cells served as negative control and allowed setting of the fluorescence cutoff (vertical line in flow cytometry density plots, Fig. 2B).

2.8. Treatment of MDM with recombinant IL-1α (rIL-α) and recombinant IL-1β (rIL-1β)

Bovine MDM from healthy cattle ($n = 3$–5) were cultured in RPMI 1640 tissue culture medium without phenol red supplemented with 10% fetal bovine serum and stimulated with rIL-1α or rIL-1β (R&D systems, Minneapolis, MN) at different concentrations of 0, 5, 25, 50, and 100 ng/ml for 24 h or the indicated times in

time course studies. Protein lysates were collected in RIPA buffer and stored at −80 °C until use in Western blotting. RNA was extracted as described under quantitative real time RT-PCR and stored at −80 °C until use.

2.9. Immunoprecipitation Western blotting of TRAF1

TRAF1 protein detection in MDM cells by traditional Western blotting was difficult due to a low level of expression. We therefore employed an immunoprecipitation (IP)–Western blot procedure, using two different anti-TRAF1 antibodies for IP and Western blot detection. Immunoprecipitation was performed using a 50% slurry of Protein G Sepharose 4 Fast Flow (Protein G Sepharose; Amersham Biosciences, Pitscataway, NJ) washed and prepared essentially as recommended by the manufacturer. After the final wash, 250 μl of RIPA buffer was added to the remaining 250 μl of Protein G Sepharose (50% slurry).

Cell lysates (100 μg total protein/ml) were precleared by adding 50 μl of 50% Protein G Sepharose slurry to 1 ml of cell lysate in a 1.5 ml snap-cap tube. Tubes were then incubated at 4 °C for 1 h on a rotating mixer. After incubation, tubes were centrifuged at 12,000 rpm for 20 s to pellet the Protein G Sepharose. Supernatants were removed to a new tube and immediately used for immunoprecipitation with 2 μl of anti-TRAF-1 monoclonal antibody (sc-6253, Santa Cruz Biotechnologies, Santa Cruz, CA.) and 50 μl of washed Protein G Sepharose. Samples were incubated at 4 °C overnight on a rotating mixer. After overnight incubation, samples were centrifuged at 12,000 rpm for 5 min at 4 °C to collect immune complexes on the Protein G Sepharose beads. Supernatants were carefully removed and discarded. To remove unbound proteins, 500 μl of RIPA buffer was added to each sample and mixed gently. Samples were centrifuged at 12,000 rpm for 5 min at 4 °C and the supernatant was carefully removed. Washes were repeated three additional times. Finally, 20 μl of prewarmed (56 °C) 5 × SDS sample buffer and 1 μl of a 1:1 mixture of bromophenol blue and beta-2-mercaptoethanol was added to each sample. Proteins were denatured in a heating block set to 90 °C for 10 min and the Protein G Sepharose beads pelleted by centrifugation. Supernatants containing proteins were carefully removed and the proteins were separated on 10% SDS-PAGE gels. Proteins were electrophoretically transferred onto nitrocellulose membranes (Amersham Bioscience Inc.). TRAF1 protein was detected by Western blot analysis essentially as described (Aho et al., 2003). Briefly, membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) containing 0.2% Tween 20 (0.2% TBST) for 1 h at room temperature. Blocked membranes were probed with anti-TRAF1 rabbit polyclonal antibody (1: 1000 dilution, sc-1830; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Membranes were washed in 0.2% TBST three times for 15 min each, followed by a 1 h incubation with mouse anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:10,000 dilution, sc-2357; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature. After a final 2 washes with 0.2% TBST for 15 min each, protein bands were visualized using the SuperSignal West Pico Kit (Pierce Endogen Inc., Rockford, IL), followed by exposure to X-ray film (Amersham Bioscience Inc.).

2.10. IL-1α ELISA assay and TRAF1 Western blotting

To measure IL-1α production, primary bovine MDM were infected with MAP at a MOI of 10:1 (10 bacilli/macrophage) in 96-well culture dishes, as described above. Nil controls consisted of MDM cells to which no bacteria were added. At 0, 0.5, 1, 2, 3, 6, 12, and 18 h after the addition of bacteria, medium was removed and collected for IL-1α assay using a cross-reactive IL-1α ELISA assay Kit (Pierce Endogen Inc.), essentially according to the manufacturer's protocol. MDM remaining in wells following removal of culture media were lysed with RIPA buffer and collected for immunoprecipitation and Western blot analysis as described above.

2.11. RNA extraction and quantitative real time reverse transcriptase polymerase chain reaction (Q-RT-PCR)

RNA was extracted from MDM cells using Trizol reagent (Invitrogen Life Technologies Corp., Carlsbad, CA) as described previously (Coussens et al., 2004, 2005). All RNA samples were treated with RNase-free DNase I (Promega Corp., Madison, WI). Quality and quantity of extracted total RNA was estimated using an Agilent Bioanalyzer 2100 (Agilent Corp., Foster City, CA). Q-RT-PCR analyses were performed in an Applied Biosystems 7000 DNA sequence detection system (Perkin-Elmer Corp., Foster City, CA), essentially as described previously (Coussens et al., 2004, 2005). Briefly, total RNA extracted from MDM cell samples was converted into first-strand cDNA by adding 2 μg of total RNA to a 12 μl reaction containing 10 mM Oligo

$(dT)_{15–18}$ primer. Following a 5 min incubation at 70 °C, the reaction was quick-chilled to 20 °C and adjusted by addition of 4 μl of a 5× buffer supplied by the RT manufacturer (final reagent concentrations were: 50 mM Tris–HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$), 1 mM dNTPs, 200 units of Superscript II RNase H^- Reverse Transcriptase (Invitrogen Life Technologies), and a final concentration of 10 mM DTT in a total reaction volume of 20 μl. The RT reaction was allowed to progress at 42 °C for 60 min, heated to 70 °C for 15 min, and cooled to 37 °C prior to the addition of 2 units of DNase-Free RNaseH (Invitrogen Life Technologies). Incubation at 37 °C continued for 20 min in the presence of RNaseH to remove original RNA templates. RNaseH was subsequently inactivated by heating at 70 °C for 10 min. First-strand cDNAs were purified by extraction with Quick-Clean Resin (BD Biosciences Inc., Alameda, CA) and precipitation in ethanol. Final cDNA pellets were suspended in 52 μl of RNase-Free ddH_2O. Concentration of cDNA in each sample was determined by Nanodrop 1000 spectrometry (Nanodrop Technologies Inc., Wilmington, DE) and adjusted with RNase-Free ddH_2O to a final working concentration of 10 ng/μl. All cDNA dilutions were stored at −80 °C until use in Q-RT-PCR reactions.

Q-RT-PCR was performed using the SYBR Green PCR Master Mix (Perkin-Elmer Corp.), 20 ng of template cDNA, and gene-specific primers. All primers were designed using Primer Express Software (Perkin-Elmer Corp.) and were synthesized using a commercial facility (Operon Technologies, Alameda, CA). Sequences used for primer design were obtained from public databases (Genbank, NCBI) as full-length cDNA sequences when available. In cases where no bovine cDNA sequence was available, EST sequences derived from the *Bos taurus* (bt) gene index (GI) from The Institute for Genome Research (TIGR) and corresponding to the target gene were used. Primer sequences are posted at (www.cafg.msu.edu) under the ''primers'' section along with additional information including expected T_m of products and appropriate primer concentration ratios.

All reactions were performed in duplicate and Q-RT-PCR data was analyzed using the $2^{-(\Delta\Delta Ct)}$ method as described (Livak and Schmittgen, 2001). To assess the effect of MAP infection, rIL-1α treatment, or rIL-1β treatment of MDM cells, β-actin served as the control gene (calculation of ΔCt) and uninfected cells (MAP infection) or untreated cells (rIL-1α and rIL-1β treatment) served as the calibrator (calculation of ΔΔCt).

2.12. Statistical analysis

All results were compared by using one-way analysis of variance, and significant differences between means of treatments were tested by the Fisher protected least significant difference test (SAS/STAT, 1996). For all tests and comparisons, only *p*-values less than 0.05 were considered significant.

3. Results

3.1. Enhanced levels of TRAF1 protein within macrophages infiltrating MAP-associated lesions

Immunohistochemical staining procedures were used to determine which cells in lesions from MAP infected

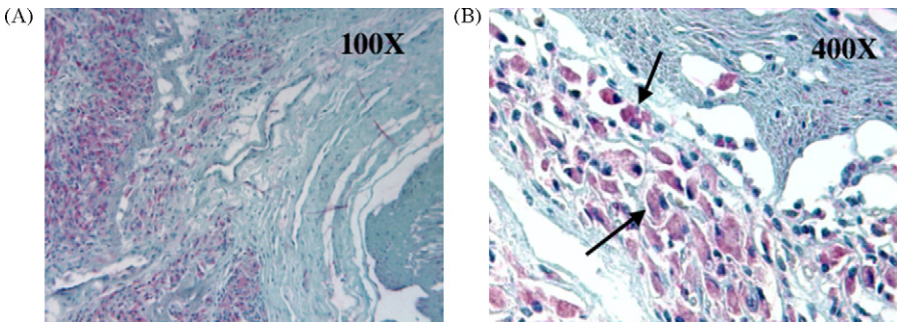

Fig. 1. Representative immunohistochemical staining of ileal tissue from a cow with clinical Johne's disease. Tissues were isolated and processed for staining as described in Section 2 using a TRAF1 specific rabbit polyclonal primary antibody (Santa Cruz Biotechnology, #sc-7186). Antibody binding was detected using a mouse anti-rabbit IgG monoclonal secondary antibody conjugated to alkaline phosphatase (AP) and development using an enhanced AP red kit (Ventana Inc., Tucson, AZ). Controls included infected tissues with secondary antibody only and tissues from uninfected control cattle. All controls slides were negative for staining (data not shown). Sections were examined and photographed on a Leica model DMIL inverted fluorescent microscope outfitted with a 5. 1 Mpixel Leica DCF480 digital camera. Images are shown at 100× (Panel A) and at 400× (Panel B). Representative macrophages stained positive for TRAF1 are highlighted by black arrows.

cattle contained enhanced levels of TRAF1 observed previously (Aho et al., 2003). Acid-fast staining revealed high numbers of MAP within macrophages in ileal sections from Johne's disease positive cattle and, as expected, the absence of MAP in sections from uninfected cattle (data not shown). Serial sections from ileal regions with large numbers of acid-fast staining bacteria revealed high levels of TRAF1 protein, consistent with previous Western blot analyses (Aho et al., 2003) (Fig. 1). Specifically, increased TRAF1 protein expression was observed localized within macrophage cells infiltrating ileal tissue from all three Johne's disease positive cattle (Fig. 1). Cell-specific staining for IL-1α could not be detected, as the IL-1α staining pattern was highly diffuse, consistent with secretion of IL-1α from surrounding cells (data not shown).

3.2. Characterization of in vitro differentiated bovine MDM cells

Because immunohistochemical evidence suggested that macrophages were the predominate source of enhanced TRAF1 protein in lesions from Johne's disease positive cattle, we next wished to investigate the link between MAP infection, TRAF1, and IL-1α expression in isolated bovine macrophage cells in vitro. However, prior to conducting these studies, we wished to characterize in vitro differentiated bovine

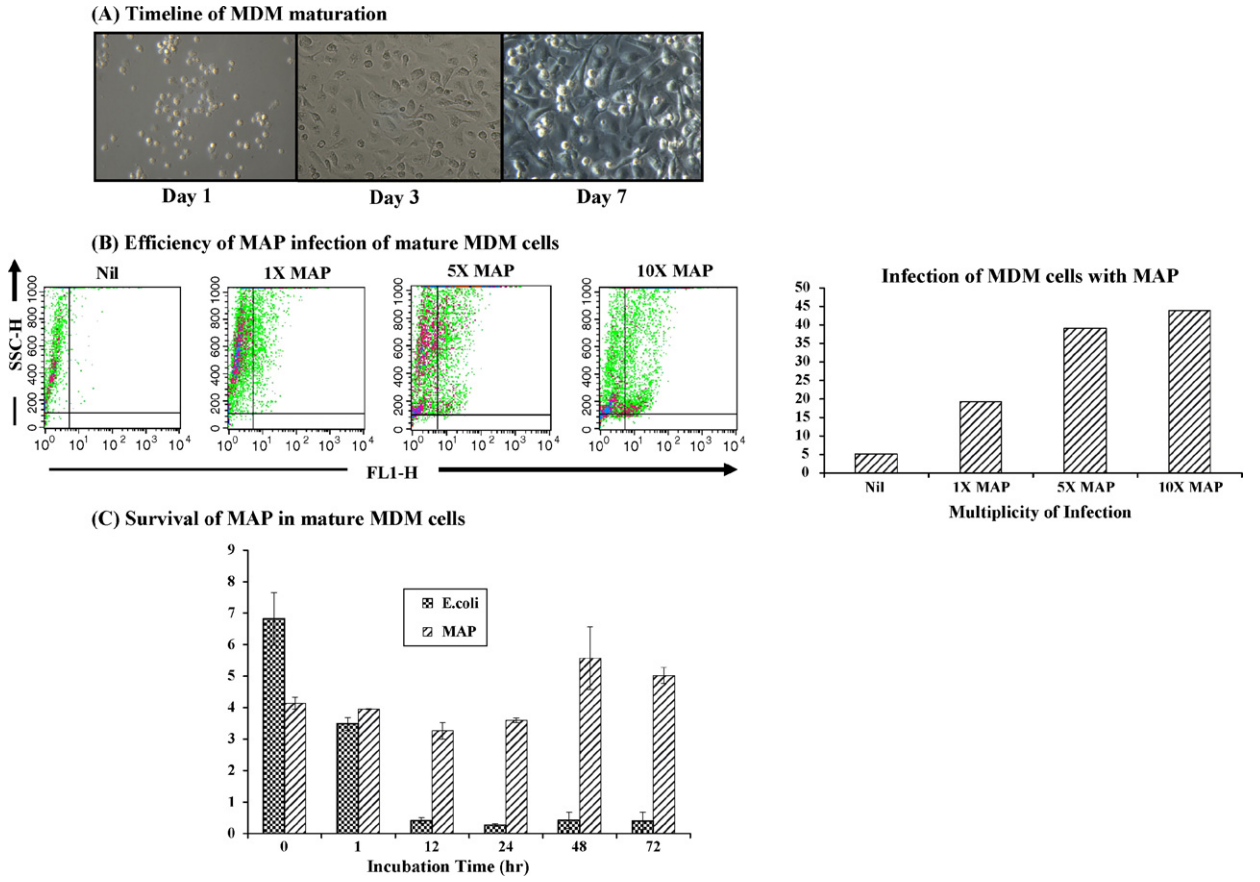

Fig. 2. Initial characterization of bovine monocyte-derived macrophages (MDM). Primary bovine monocytes were isolated as described in Section 2 and cultured in RPMI media supplemented with 10% fetal bovine serum (complete RPMI). Panel A: Representative photomicrographs of MDM cells at days 1, 3, and 7 of culture. By day 7, cells were morphologically similar to mature macrophages with a large flattened appearance except for a rounded nucleus and extensive pseudopodia. Panel B: Efficiency of MAP infection of primary bovine MDM cells following in vitro maturation. Cells were prepared, cultured, and infected with various multiplicities of infection with a green fluorescent protein expressing strain of MAP as described in Section 2. Following extensive washing at 2 h post-infection to remove uningested bacteria, cells were incubated in complete RPMI media for an additional 24 h, prior to analysis of GFP content by flow cytometry. Panel C: Survival of MAP and *E. coli* in primary bovine MDM cells matured in vitro. Cells were prepared and cultured as described in Section 2 prior to infection with either *E. coli* or MAP. At the indicated times post infection, cells were harvested and total DNA isolated, also as described in Section 2. MAP DNA was detected using primers specific for the IS900 insertion element and *E. coli* DNA was detected using primers specific for the 16S ribosomal gene.

MDM cells in terms of morphological features, phagocytosis of bacteria, and survival of MAP. Bovine MDM cells were isolated and allowed to differentiate in vitro as described in Section 2. On day one of culture adherent monocytes were characteristically small and rounded (Fig. 2, Panel A). After three days in culture, the cells were morphologically similar to immature macrophages, with considerable expansion of cytoplasm and early pseudopodia (Fig. 2, Panel A). By day 7 of culture, MDM cells had morphological characteristics consistent with mature macrophages, including a large flattened appearance, abundant pseudopodia, and a rounded nucleus (Fig. 2, Panel A). These results were consistent with previous literature on differentiation of bovine MDM cells (Woo et al., 2005).

To examine phagocytosis of MAP by mature MDM cells, the cells were infected with different amounts of a green fluorescent protein (GFP) expressing strain of MAP (a K10 derivative, generous gift from R. Barletta, University of Nebraska, Lincoln, NE) as described in Section 2 (MOIs of 1, 5, 10, and 20). Cells were collected 2 h post-infection and subjected to flow cytometry to measure the percent of cells infected with GFP-MAP. As shown in Fig. 2, Panel B, mature MDM cells readily took up MAP at all MOIs, but the maximum level of infection was approximately 45% of total cells at a MOI of 10. Increasing the MOI beyond this level to 20× had little effect on the percent of cells showing positive for GFP (data not shown). Although there was a general reduction in side-scatter in the 10× MAP infected cells, there were no obvious morphological differences in cells exposed to 1×, 5× or 10× MAP as determined by light microscopy and we have previously shown that 10× MAP infection does not increase the number of apoptotic cells relative to cells exposed to lower doses of bacteria (Sommer et al., unpublished observations).

Finally, we wished to examine the survival of MAP in mature MDM cells, relative to a bacterium that should be readily destroyed in functional macrophages. To accomplish this, mature MDM cells were infected with either MAP or a laboratory strain of *E. coli* at a MOI of 10. At various times post-infection, cells were lysed and total DNA extracted. Presence of MAP DNA was determined by quantitative-PCR using primers specific for the IS900 insertion element while presence of *E. coli* DNA was detected using primers specific for the 16S ribosomal gene. As shown in Fig. 2, Panel C, *E. coli* was readily destroyed in MDM cells while MAP persisted at levels similar to time 0 (after an initial 2 h incubation and just subsequent to washing to remove non-ingested bacteria), even out to 72 h post-infection. Taken together, these results suggested that mature MDM cells had a morphological appearance consistent with a macrophage phenotype, that the cells were capable of readily

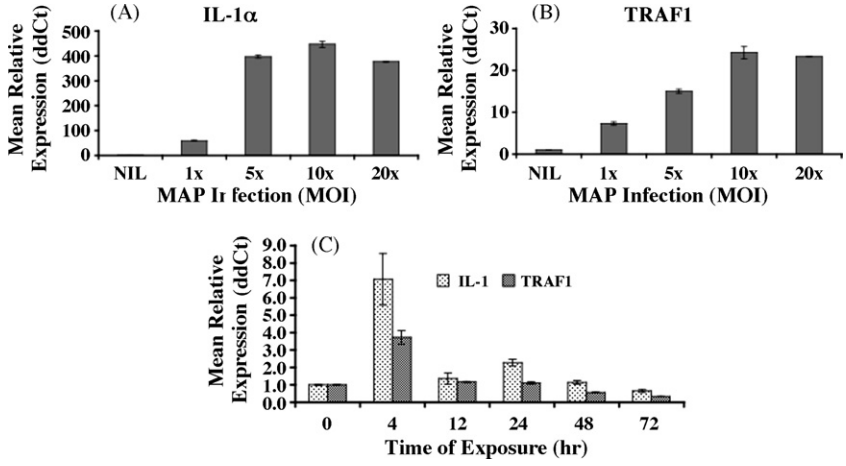

Fig. 3. Enhanced expression of IL-1α and TRAF1 mRNA following infection of primary bovine MDM cells, as measured by Q-RT-PCR. Primary bovine MDM cells were isolated, cultured for seven days, and infected with MAP at various MOI from 1 to 10 as described in Section 2. Cells were harvested at the indicated times post-infection and RNA isolated for Q-RT-PCR. RNA processing, cDNA conversion, and Q-RT-PCR analysis were also performed as detailed in Section 2. Panel A: Expression of IL-1α mRNA in MDM cells 6 h post-infection with various MOI of MAP. Data is expressed relative to IL-1α mRNA expression in uninfected cells. Panel B: Expression of TRAF1 mRNA in MDM cells 6 h post-infection with various MOI of MAP. As with IL-1α mRNA, data is expressed relative to TRAF1 mRNA expression in uninfected cells. Panel C: Time course of IL-1α and TRAF1 mRNA expression following infection of MDM cells with MAP at a MOI of 10. For each time point, the mean (±S.E.M.) of MDM cells from five donor cattle is shown.

phagocytosing MAP, and that the cells were capable of readily destroying susceptible bacteria, but not MAP.

3.3. MAP infection of MDM enhances expression of IL-1α and TRAF1 mRNA

Once we were confident that MDM cells were a reasonable system in which to study MAP-macrophage interactions in vitro, we next wished to determine if infection of MDM cells by MAP would lead to enhanced levels of IL-1α and TRAF1 mRNA. In initial studies, infection of MDM cells with MAP enhanced expression of both TRAF1 and IL-1α mRNA expression in a dose dependent manner up to a MOI of 10. Increasing the amount of input MAP beyond this level did not appreciably enhance expression of either the TRAF1 or IL-1α genes (Fig. 3, Panels A and B). We therefore used a MOI of 10:1 (MAP to cells) for additional investigations. We next performed an infection time course, extracting cellular RNA at 0, 4, 12, 24, 48, and 72 h post-infection with MAP at a MOI of 10. Q-RT-PCR analysis revealed that MAP infection enhanced both IL-1α (seven-fold) and TRAF1 (four-fold) mRNA levels by 4 h of infection, relative to uninfected cells, but that elevated expression levels of both genes had largely declined to pre-infection status by 12 h post infection (Fig. 3, Panel C).

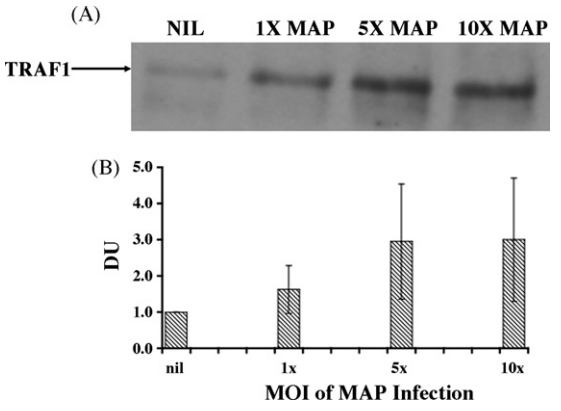

Fig. 4. Infection of primary bovine MDM cells with various MOI of MAP enhances expression of TRAF1 protein in a dose dependent manner. Primary bovine MDM cells were isolated, cultured to day seven, and infected as described in Section 2. At 24 h post-infection, cells were harvested in RIPA buffer and processed for immunoprecipitation (IP)–Western blot analysis as detailed in Section 2. Panel A depicts a representative IP–Western blot for TRAF1 protein and Panel B depicts the mean results (±S.E.M.) of MDM cells from four donor cattle.

3.4. TRAF1 mRNA and protein expression increases in MDM following infection with MAP

To determine if the observed increase in TRAF1 mRNA following infection of MDM cells with MAP led to increased TRAF1 protein, we performed immunoprecipitation (IP)–Western blot assays as described in Section 2. MDM were infected with MAP at a MOI of 1, 5, or 10 and infection allowed to progress for 24 h. Uninfected MDM served as a negative infection control for baseline TRAF1 protein levels. Cell lysates were collected and proteins extracted for IP–Western blot analysis, as described in Section 2. IP–Western Blot analysis revealed that MAP significantly increased TRAF1 protein expression in bovine MDM ($p = 0.0155$ at a MOI of 5 and $p = 0.0029$ at a MOI of 10) (Fig. 4). Expression of TRAF1 in cultured bovine MDM was dependent upon MOI, and was positively correlated with amount of MAP added to cultures (R value = 0.7543; $p = 0.0007$). Relative to uninfected cells, mean TRAF1 protein expression in infected MDM was over 2-fold greater in cells exposed to MAP at a MOI of 5 and over 2.5-fold greater in cells exposed to MAP at a MOI of 10 (Fig. 4).

3.5. MAP infection of MDM enhances expression of IL-1α

Given that macrophages were a likely source of enhanced IL-1α protein levels in lesions from MAP-infected cattle (Aho et al., 2003) and MAP infection of MDM cells led to increased abundance of IL-1α mRNA, we next wished to determine if MAP could induce IL-1α protein production in cultured bovine MDM. To accomplish this, MDM cells were infected

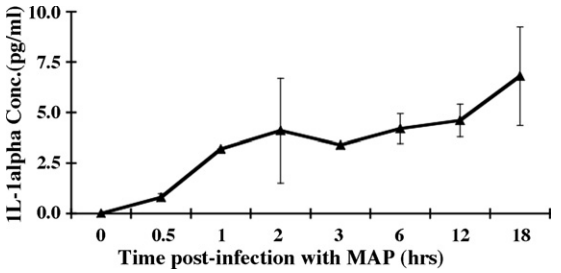

Fig. 5. Infection of primary bovine MDM cells with MAP enhances secretion of IL-1α protein into culture supernatants. Primary bovine MDM cells were isolated, cultured to day seven, and infected with MAP as described in Section 2. Medium was collected from cultures in 96-well plates (8 wells/time point) and processed for IL-1α ELISA also as described in Section 2. Results presented are the mean (±S.E.M.) of data from MDM cells representing two different donor cattle.

with MAP at a MOI of 10 and, at various times post-infection, media was removed from separate cultures and concentration of IL-1α secreted into the media monitored by ELISA as described in Section 2. Uninfected MDM in culture will produce small amounts of IL-1α that can accumulate over time. To account for this at each time point, the amount of IL-1α in uninfected control cultures was subtracted from the amount found in infected MDM. Results from this time course study indicated that indeed cultured bovine MDM infected by MAP displayed enhanced IL-1α production relative to uninfected cells (Fig. 5). In fact, at 12 h post-infection with MAP, media from cultures of infected MDM contained two-fold greater amounts of IL-1α than media from nil treated MDM ($p = 0.0413$). Thus, infection of MDM with MAP enhanced expression of both TRAF1 and IL-1α in a dose and time dependent manner, respectively (Figs. 4 and 5).

3.6. IL-1α alone enhances TRAF1 protein expression in primary bovine MDM

One consequence of IL-1α stimulation is activation of nuclear factor kappa B (NFκB) (Li et al., 2001; Wolf et al., 2001) and TRAF1 expression is, at least in part, regulated by NFκB (Bradley and Pober, 2001). Therefore, we wished to determine if TRAF1 expression could be enhanced by rIL-1α in normal uninfected bovine MDM. Indeed, IP–Western blot analysis revealed that TRAF1 protein expression increased in bovine MDM following 12 h treatments with rIL-1α at 5, 25, 50, and 100 ng/ml in a dose dependent manner (R value = 0.7455; $p < 0.0001$)

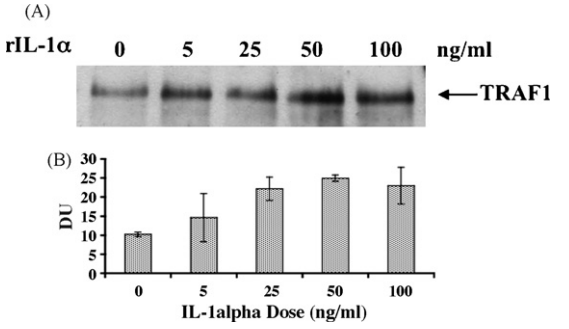

Fig. 6. IL-1α alone enhances expression of TRAF1 protein in primary bovine MDM cells. Primary bovine MDM cells were isolated and cultured to day seven as described in Section 2. On day seven, cells were treated with recombinant human IL-1α (R&D Systems, Minneapolis, MN) at the indicated concentrations. Following 24 h of treatment, cells were lysed in RIPA buffer and processed through IP–Western blot as described in Section 2. In Panel A, a representative IP–Western blot is shown and in Panel B the mean (±S.E.M.) for three biological replicates is plotted.

(Fig. 6). Mean TRAF1 protein expression in MDM treated with 50 ng/ml rIL-1α was over 2.5-fold greater than expression in Nil-stimulated control MDM (Fig. 6).

3.7. Effect of MAP and rIL-1α receptor antagonist on TRAF1 protein expression in bovine MDM

In order to clarify the role of IL-1α on TRAF1 protein expression in MAP infected bovine MDM, we next used rIL-1 receptor antagonist (rIL-1RA) to block IL-1 receptors prior to infection with MAP and followed production of TRAF1 protein over time. TRAF1 protein levels increased in MAP infected MDM cells at the same rate regardless of rIL-1RA presence up to 3 h post-infection. By 6 h post-infection however, cells pre-treated with rIL-1RA contained significantly less TRAF1 protein ($p = 0.0016$) than cells not treated with rIL-1RA (Fig. 7). By 12 h post-infection, TRAF1 protein levels in infected MDM were significantly higher than in either uninfected MDM or in infected MDM that had been pre-treated with rIL-1RA ($p = 0.0092$).

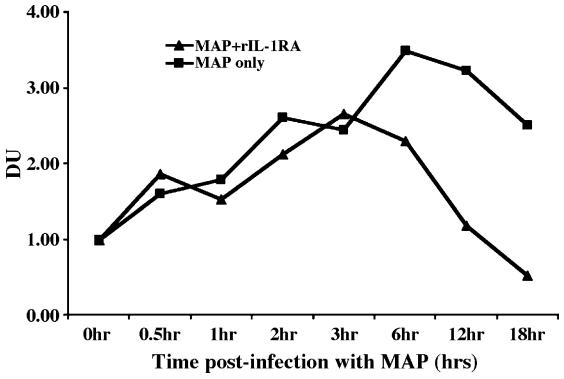

Fig. 7. Sustained expression of TRAF1 protein in MAP infected MDM cells depends upon interaction of IL-1 with its receptor. Primary bovine MDM cells were isolated and cultured to day seven as described in Section 2. On day seven of culture, cells were treated with recombinant IL-1 receptor antagonist (rIL-1RA) at 100 pg/ml (▲) or PBS (■). After 30 min of pre-incubation with rIL-1RA, cells were infected with MAP at a MOI of 10. At the indicated times post-infection, cells were lysed with RIPA buffer and processed through IP–Western blotting to detect TRAF1 as described in Section 2. The final chemiluminescent images were processed and analyzed on a scanning densitometer. DU refers to arbitrary densitometric units. Uninfected MDM cells in culture served as controls at each time point. Since MDM cells make small but measurable amounts of TRAF1, the control value, was subtracted from the infected culture values at each time point. A representative result from a series of three blots is shown. For each replicate, MDM cells from different cows were used. Although variances in DU were high between biological replicates, the trend was similar in all cases.

3.8. MAP infection of MDM cells increases expression of IL-1β mRNA and IL-1β increases expression of TRAF1 in uninfected MDM cells

It was clear from our data that rIL-1α alone could cause a dose and time dependent increase in TRAF1 mRNA and protein expression in MDM cells in the absence of MAP infection. However, both the effect of MAP infection on IL-1β expression and the effect of IL-1β on TRAF1 expression were not clear. Since IL-1α and IL-1β utilize the same cellular receptor they would presumably cause similar signaling cascades within most cells. Our results with IL-1RA could not distinguish effects of IL-1α and IL-1β and it remained possible that at least some of the increase in TRAF1 expression observed in MDM cells following MAP infection were due to IL-1β. To begin to address this issue, we infected MDM cells with MAP at various MOI, isolated RNA at 6 h post-infection and examined expression of IL-1β mRNA by Q-RT-PCR. MAP caused a rather dramatic increase in IL-1β gene expression, relative to uninfected MDM cells (Fig. 8A). The increase in IL-1β gene expression did not appear to be as dose dependent as that observed with IL-1α. Next, we wished to determine if IL-1β alone could increase expression of TRAF1 mRNA, similar to what had been observed for IL-1α. Indeed, addition, of IL-1β in increasing concentrations to MDM cell cultures enhanced expression of TRAF1 mRNA in a dose dependent manner (Fig. 8B).

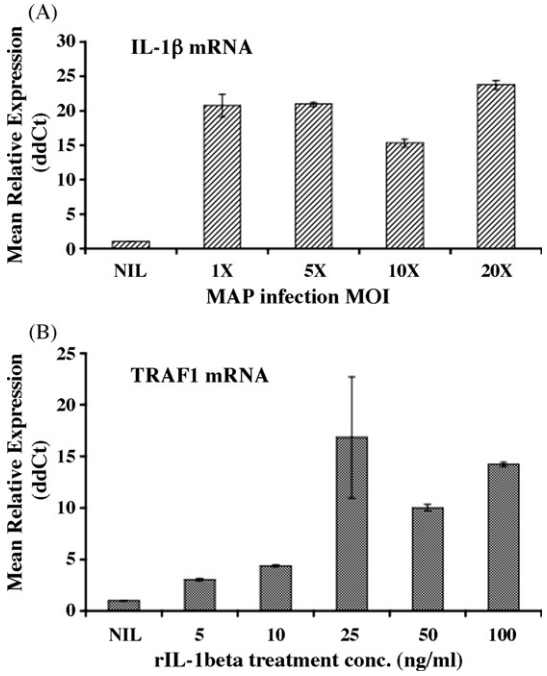

Fig. 8. MAP infection of primary bovine MDM cells enhances IL-1β mRNA expression and recombinant IL-1β alone enhances TRAF1 mRNA expression in primary bovine MDM cells. Primary bovine MDM cells were isolated and cultured to day seven as described in Section 2. Panel A: On day seven of culture, cells were infected with MAP at various MOI, as indicated. Infections were allowed to proceed for 6 h before cells were harvested and processed for RNA isolation as described in Section 2. Expression of IL-1β mRNA was assessed by Q-RT-PCR, also as described in Section 2. Data was analyzed by the $2^{-(\Delta\Delta Ct)}$ method as described previously (10, 21). Panel B: On day seven of culture, primary bovine MDM cells were treated with the indicated concentrations of recombinant human IL-1β (R&D Systems, Minneapolis, MN) for 6 h. Cells were harvested and processed for RNA extraction and Q-RT-PCR to detect TRAF1 mRNA abundance as described in Section 2. Data was analyzed by the $2^{-(\Delta\Delta Ct)}$ method as described previously (10, 21).

4. Discussion

A previous study by our group demonstrated enhanced levels of TRAF1 and IL-1α mRNA and protein expression in lesions of ileal tissues associated with MAP infection in cattle (Aho et al., 2003). In this report, we now provide evidence that macrophage cells within lesions associated with Johne's disease in cattle are primarily responsible for previously observed high levels of TRAF1 protein. Although cell-specific staining of IL-1α was not observed in infected ileal sections, a diffuse pattern of staining, consistent with IL-1α being secreted was observed. In light of our results and the difficulty in staining secreted cytokines, it may therefore be more meaningful in future studies to determine the actual level of IL-1α in ileal sections by ELISA.

After immunohistochemistry analyses identified macrophages as the primary source for enhanced levels of TRAF1 protein found in MAP infected ileal tissues and, given that it was reasonable to assume that macrophages were also a major source of IL-1α within these same lesions, we next investigated a potential link between these two proteins using in vitro studies on cultured bovine MDM cells. These studies clearly demonstrated that: (1) infection of cultured bovine MDM with various MOI of live MAP results in a dose dependent increase in TRAF1 mRNA and protein expression relative to uninfected cultures. (2) Infection of cultured bovine MDM with MAP results in an enhanced accumulation of secreted IL-1α protein. (3) Stimulation of cultured bovine MDM with IL-1α alone enhances TRAF1 protein expression in a dose dependent manner. (4) Preincubation of cultured primary bovine MDM with rIL-1RA prevents the sustained increase in TRAF1 protein expression following infection with MAP, although an early

increase in TRAF1 protein expression is still observed. (5) Finally, IL-1β mRNA abundance is also enhanced upon MAP infection of MDM cells and IL-1β alone can enhance TRAF1 protein expression. Given these facts, we conclude that MAP infection of macrophages induces expression of IL-1α and IL-1β and enhanced expression of these cytokines is responsible for the observed sustained increase in expression of TRAF1 protein. We interpret results from IL-1RA studies to indicate that an initial increase in TRAF1 protein expression may be caused directly by MAP, perhaps through interaction with cell surface receptors, such as the Toll-like receptor system. Given that high concentrations of IL-1 are toxic and that TRAF1 would be expected to limit signaling from TNF receptor superfamily members (i.e. Fas, TNFRl, and CD40), our results have significant implications for the pathology of Johne's disease in cattle, may help to explain how MAP-infected macrophages persist within lesions associated with the disease, and may also provide clues as to why MAP-infected macrophages fail to properly activate and destroy intracellular MAP. One caveat to these hypotheses is that we detected only picogram levels of IL-1 produced by MDM cells following MAP infection and our IL-1 stimulation studies used nanogram levels of this cytokine. It will thus be important to determine the actual levels of IL-1 at sites of MAP infection in cattle afflicted with Johne's disease.

In humans, chronic enterocolitis is associated with accumulations of macrophages in inflamed regions and these same regions contain high levels of IL-1α (Viscardi et al., 1997). Assuming our results are transferable across species, one would expect to find high levels of TRAF1 protein in macrophages associated with chronic enterocolitis. Curiously, IL-1α has been shown to function in recruitment of leukocytes to sites of infection and participates, together with TNFα, in maintenance of granulomas in other mycobacterial infections (Juffermans et al., 1998, 2000). However, despite abundant expression of IL-1α and IL-8 there is a conspicuous lack of neutrophils and lymphocytes in lesions associated with clinical MAP infection and TNFα expression is not significantly elevated in MAP-induced lesions (Coussens et al., 2004). The reasons for this apparent discrepancy remain to be examined.

In other species, TRAF1 is a highly anti-apoptotic protein, preventing cell death signaling via TNF receptor superfamily members, particularly Fas (CD95) and TNFRl (Fotin-Mleczek et al., 2004). TRAF1 can also interfere with normal macrophage activation, particularly via the CD40/CD40 ligand system (Fotin-Mleczek et al., 2004). The ability of TRAF1 to interfere with these systems is linked to the ratio of TRAF1 and TRAF2 in cells, where enhanced expression of TRAF1 would tend to promote development of trimeric complexes containing either 2 or 3 TRAF1 molecules. In mice, it is well established that these complexes cannot transfer cell death signals from either TNFR1 or CD95 and are deficient in transferring signals from CD40 (Fotin-Mleczek et al., 2004). However, these complexes are capable of transferring signals from the pro-survival TNF receptor superfamiliy member TNFR2. Thus, assuming TRAF1 functions in the bovine as it does in mice, two expected consequences of enhanced TRAF1 expression would be resistance to externally triggered apoptosis and failure to properly activate following engagement of CD40 on macrophages by T cells expressing CD40 ligand. Both of these effects would be beneficial to an intracellular pathogen such as MAP. Clearly, our results suggest studies aimed at determining the relative sensitivity of MAP infected MDM cells to CD40 ligand mediated activation and to apoptosis via TNFR superfamily member ligands (i.e. Fas) are warranted. In addition, further studies using TRAF1 expression vectors could help uncouple TRAF1-specific effects from other consequences of MAP infection.

Acknowledgements

The authors wish to thank Ms. Xiaoning Ren, and Ms. Sue Sipkovsky for outstanding technical assistance, training and support. We would also like to acknowledge the many helpful discussions with colleagues including Drs. Jeanne Burton, Srinand Sreevatsan, and Bill Davis. Funding for research presented in this report was provided through the USDA-APHIS (grant # 05-9126-0993-GR) and the USDA-NRI (grant # 2005-35204-16474) and through the Michigan Agricultural Experiment Station. The Center for Animal Functional Genomics at Michigan State University is supported through a grant from the MSU Foundation.

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