Immunomodulatory effects of tulathromycin on apoptosis, efferocytosis, and proinflammatory leukotriene B_4 production in leukocytes from *Actinobacillus pleuropneumoniae*– or zymosan-challenged pigs

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OBJECTIVE
To investigate the anti-inflammatory and immunomodulatory properties of tulathromycin in vitro and in experimental models of *Actinobacillus pleuropneumoniae*–induced pleuropneumonia and zymosan-induced pulmonary inflammation in pigs.

ANIMALS
Blood samples from six 8- to 30-week-old healthy male pigs for the in vitro experiment and sixty-five 3-week-old specific pathogen–free pigs.

PROCEDURES
Neutrophils and monocyte-derived macrophages were isolated from blood samples. Isolated cells were exposed to tulathromycin (0.02 to 2.0 mg/mL) for various durations and assessed for markers of apoptosis and efferocytosis. For in vivo experiments, pigs were inoculated intratracheally with *A pleuropneumoniae*, zymosan, or PBS solution (control group) with or without tulathromycin pretreatment (2.5 mg/kg, IM). Bronchoalveolar lavage fluid was collected 3 and 24 hours after inoculation and analyzed for proinflammatory mediators, leukocyte apoptosis, and efferocytosis.

RESULTS
In vitro, tulathromycin induced time- and concentration-dependent apoptosis in neutrophils, which enhanced their subsequent clearance by macrophages. In the lungs of both *A pleuropneumoniae*– and zymosan-challenged pigs, tulathromycin promoted leukocyte apoptosis and efferocytosis and inhibited proinflammatory leukotriene B_4 production, with a concurrent reduction in leukocyte necrosis relative to that of control pigs. Tulathromycin also attenuated the degree of lung damage and lesion progression in *A pleuropneumoniae*–inoculated pigs.

CONCLUSIONS AND CLINICAL RELEVANCE
Tulathromycin had immunomodulatory effects in leukocytes in vitro and anti-inflammatory effects in pigs in experimental models of *A pleuropneumoniae* infection and nonmicrobial-induced pulmonary inflammation. These data suggested that in addition to its antimicrobial properties, tulathromycin may dampen severe proinflammatory responses and drive resolution of inflammation in pigs with microbial pulmonary infections. (*Am J Vet Res* 2015;76:507–519)

Self-perpetuating inflammation contributes to the pathogenesis of *Actinobacillus pleuropneumoniae* infection in swine.[1] Excessive extravasation of neutrophils and their subsequent uncontrolled death at the site of inflammation can lead to local release of proteolytic enzymes, reactive oxygen species, and potent proinflammatory mediators that exacerbate the inflammatory response and amplify the degree of tissue injury.[2] Leukotriene B_4, a proinflammatory lipid mediator derived from arachidonic acid, acts as a potent neutrophil chemoattractant and is an important marker of inflammation.[3,4] Similarly, the release of LDH is a prominent marker of cellular necrosis and hallmark of pulmonary tissue damage[5] caused by *A pleuropneumoniae* in swine.

In homeostatic conditions, infiltrating immune cells undergo programmed cell death (apoptosis) and

ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
HBSS	Hanks balanced salt solution
HI-FBS	Heat-inactivated fetal bovine serum
IL	Interleukin
LDH	Lactate dehydrogenase
LTB_4	Leukotriene B_4
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor κB
TUNEL	Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling

are subsequently cleared from the tissues by resident macrophages.[6] During apoptosis, senescent cells become desensitized to inflammatory stimuli and cellular organelles are disassembled and degraded within apoptotic bodies while cellular membranes remain intact; this prevents release of injurious cellular contents into the extracellular environment.[7,8] Apoptotic bodies then signal for their own nonphlogistic clearance by resident macrophages.[9–11] In situations of severe inflammation, excessive recruitment of neutrophils can overwhelm homeostatic clearance mechanisms. The neutrophils then undergo necrosis, which is associated with a loss of cellular membrane integrity and the release of cytotoxic cellular contents, consequently damaging surrounding cells and perpetuating the inflammatory response.[12] This exaggerated and self-amplifying inflammatory response is common in bacterial-induced pneumonia, including infection with *A pleuropneumoniae*. Medications capable of breaking this amplification cycle and restoring homeostatic balance through resolution of inflammation would be useful in the treatment of inflammatory disease.

Actinobacillus pleuropneumoniae is a highly contagious, gram-negative coccobacillus[13,14] that is transmitted rapidly through pig herds in which adequate biosecurity is lacking. Infection with NAD-dependent strains of *A pleuropneumoniae* can result in signs of severe disease within 12 hours after exposure and in death within 24 to 36 hours thereafter.[15] Gross examination of lungs from infected pigs reveals demarcated lesions in the middle, cranial, and caudal lobes that are characterized by pulmonary consolidation, fibrosis, and necrosis.[1,16] Histologic examination of the lungs 3 hours after intratracheal inoculation with *A pleuropneumoniae* reveals alveolar edema, fibrin, and severe neutrophil infiltration.[1] Large coalescing foci of necrosis, hemorrhage, and edema can be seen 24 hours after inoculation.[1]

The vast degree of infiltration and activation of neutrophils within the lungs contributes considerably to disease progression. Indeed, treatment of pigs with indomethacin, a cyclooxygenase inhibitor capable of inhibiting neutrophil activation, prior to intratracheal inoculation with *A pleuropneumoniae* delayed development of pulmonary lesions in 1 study.[1] Those findings suggested a primary role for neutrophil activation in tissue damage associated with *A pleuropneumoniae* infection. Moreover, treatment with tilmicosin, a macrolide with proapoptotic effects in neutrophils, led to a significant decrease in the number of pulmonary lesions following experimental *A pleuropneumoniae* infection in piglets,[17] providing further evidence of a role for neutrophils in mediating necrotic tissue damage associated with infection. It follows that medications that promote apoptosis of neutrophils and their subsequent nonphlogistic removal by resident phagocytes may be particularly efficacious in the treatment of inflammatory diseases such as *A pleuropneumoniae* pleuropneumonia in swine.

Macrolide antimicrobials can modulate the host immune response[18] and thus may be useful in the treatment of infectious diseases involving a pathogenic inflammatory response. Indeed, macrolides exert several anti-inflammatory effects by inhibiting recruitment of neutrophils[19]; reducing the production of proinflammatory cytokines and chemokines such as tumor necrosis factor-β, IL-1β,[20,21] and IL-8[22–24]; preventing the activation of proinflammatory NF-κB.[25,26] Some macrolides can also promote cellular death by apoptosis,[27–31] which may provide additional benefit by contributing to the resolution of inflammation. However, the precise mechanisms underlying the anti-inflammatory and proapoptotic properties of macrolides remain incompletely understood.

Tulathromycin is a macrolide antimicrobial derived from erythromycin that is approved for use in the treatment and prevention of respiratory disease in swine and cattle. Success of tulathromycin for use in treating respiratory diseases may be attributed, at least in part, to its preferential accumulation within pulmonary tissues.[18,32] This antimicrobial has a high affinity for absorption within neutrophils and macrophages,[32,33] which aids in its rapid delivery to the site of inflammation. The anti-inflammatory and proapoptotic properties of tulathromycin have been demonstrated in bovine neutrophils[31] and macrophages,[34] and these properties may enhance the antimicrobial's efficacy in the treatment of bovine respiratory disease. Moreover, our research group has shown that tulathromycin also exerts proresolution effects (promoting the resolution of inflammation) in bovine neutrophils by influencing the production and release of lipid mediators.[35]

The purpose of the study reported here was to investigate whether tulathromycin would confer anti-inflammatory and proresolution benefits through mechanisms similar to those in cattle when used for the treatment of respiratory disease in swine. Specifically, we wanted to investigate immunomodulatory effects of tulathromycin on porcine neutrophils and macrophages in 3 complementary experiments: experimentally induced *A pleuropneumoniae* infection in young pigs, zymosan-triggered pulmonary inflammation in the absence of bacterial stimuli in young pigs, and in vitro assessment of the effects of tulathromycin on leukocytes obtained from young healthy pigs. We hypothesized that tulathromycin would exert proapoptotic effects on neutrophils and facilitate nonphlogistic removal of the dying cells by macrophage efferocytosis. Moreover, we hypothesized that tulathromycin would have additional anti-inflammatory effects in vivo by inhibiting the production of potent proinflammatory mediators such as LTB_4, as has been demonstrated in a model of bovine respiratory disease.[31] Taken together, the findings would further elucidate mechanisms through which tulathromycin may contribute to the resolution of severe inflammation that is characteristic of bacterial pleuropneumonia in swine.

Materials and Methods

Animals

Six healthy 8- to 30-week old castrated male pigs initially weighing 15 to 20 kg were obtained from a herd free of *Mycoplasma hyopneumoniae* infection and retained as blood donors to assess the effects of tulathromycin on leukocytes in vitro. Sixty-five healthy 3- to 4-week-old weaned male pigs weighing 4.5 to 11 kg were obtained from the same herd and used for in vivo experiments involving infection with live *A pleuropneumoniae* or inoculation with abiotic zymosan.

Protocol for the in vitro experiment

To obtain leukocytes for the in vitro experiment, blood samples were collected from the cranial vena cava of the healthy male pigs via 16-gauge, 2-inch needles into evacuated tubes containing 1.5 mL of citrate-dextrose solution.[a] Neutrophils were extracted and purified by means of differential centrifugation and hypotonic lysis. Briefly, blood was centrifuged at 1,200 × *g* for 20 minutes at 4°C. Plasma and buffy coat fractions were removed, and the remaining cells were washed with an equal volume of HBSS[b] without calcium chloride and recentrifuged at 1,200 × *g* for 10 minutes at 4°C. The resulting cellular pellet was resuspended in HBSS, and contaminating erythrocytes were eliminated by the addition of 20 mL of cold filter-sterilized hypotonic lysis solution (10.6mM Na_2HPO_4 and 2.7mM NaH_2PO_4). Isotonicity was subsequently restored with 10 mL of cold 3X hypertonic restoring solution (10.6mM Na_2HPO_4, 2.7mM NaH_2PO_4, and 462mM NaCl). The lysis procedure was repeated twice, and the cell pellet was resuspended in HBSS containing 10% HI-FBS[c] to optimize the cell environment. With a hemocytometer,[d] neutrophil viability was assessed on the basis of the percentage of cells that excluded 0.1% trypan blue solution.[e] Differential cell counts were performed on stained[f] cytocentrifuge[g] preparations to assess neutrophil purity. All neutrophil preparations used for in vitro experiments were assessed to be > 90% pure and > 90% viable.

For monocyte isolation and macrophage differentiation, whole blood was pooled and centrifuged at 1,200 × *g* for 20 minutes at 4°C to isolate the buffy coat. The buffy coat was diluted 1:1 with filter-sterilized saline (0.9% NaCl) solution, layered onto a polysucrose and sodium diatrizoate gradient, and centrifuged at 1,500 × *g* for 40 minutes at 4°C. The cell suspension was then collected, washed with HBSS, and centrifuged at 500 × *g* for 10 minutes at 4°C. Contaminating erythrocytes were removed by hypotonic lysis in 10 mL of cold sterile double-distilled water for 30 seconds, followed by the addition of 20 mL of cold 2X HBSS to restore isotonicity. Mononuclear cells were then resuspended in Iscove modified Dulbecco medium containing 10% HI-FBS[c] and cultured for macrophage differentiation. Cells were counted with a hemocytometer[d] and viability was assessed on the basis of the percentage of cells that excluded 0.1% trypan blue solution.[e] Differential cell counts were performed on stained[f], cytocentrifuged[g] preparations to assess monocyte purity. For macrophage differentiation, mononuclear cells (2×10^5 cells/well) were plated onto 48-well plates.[h] Cells were incubated in Iscove modified Dulbecco medium containing 10% HI-FBS for 60 minutes in a humidified incubator at 37°C with 5% CO_2 and 95% air. Nonadherent cells were removed by washing 3 times with warm (37°C) HBSS. Remaining adherent monocytes were incubated at 37°C and 5% CO_2 in Iscove modified Dulbecco medium containing 10% HI-FBS, penicillin (100 U/mL), streptomycin (100 U/mL), and tylosin (80 µg/mL) for 7 days to allow for macrophage differentiation. Culture medium was replenished every 2 to 3 days. Macrophage differentiation was > 95% pure throughout the study and > 95% mature at 7 days.

Assessment of the effects of tulathromycin on leukocytes—To assess in vitro proapoptotic effects of various concentrations of tulathromycin, purified porcine neutrophils (1×10^6 cells) were incubated with tulathromycin[i] (0.02 mg/mL to 2.0 mg/mL), staurosporine (1.0µM), or 10% HI-FBS in HBSS (control treatment) at 37°C and 5% CO_2 for 0.25 to 2 hours. Apoptosis was measured with an ELISA kit[j] in accordance with the manufacturer's instructions. The ELISA specifically measured the histone region (H1, H2A, H2B, H3, and H4) of mono- and oligonucleosomes released during apoptosis. Data were calculated from light absorbances acquired at 405 nm.[k] The TUNEL technique was used to detect apoptotic DNA fragmentation in treated neutrophils. Briefly, cytocentrifuged preparations were fixed in 4% paraformaldehyde and made permeable with 0.1% Triton X-100[l] in 0.1% sodium citrate solution. Afterward, slides were washed with PBS solution and incubated with TUNEL reaction mixture[m] at 37°C for 1 hour in the dark, in accordance with the manufacturer's instructions. The percentage of TUNEL-positive neutrophils within the cell population was determined by use of fluorescent microscopy; investigators did not have knowledge of each sample's identity. At least 100 cells/group were counted within a randomly selected field of view.

Degree of caspase fragmentation was measured in isolated neutrophils by means of western blot detection and densitometric analysis of active cleaved caspase-3 fragments normalized to β-actin. Briefly, cells obtained from each group were washed with HBSS and lysed with a lysis buffer (1% octylphenoxypolyethoxyethanol, 0.1% SDS, and 0.5% sodium deoxycholate diluted in PBS solution) containing a protease inhibitor.[n] Total protein concentrations were determined by means of a Bradford protein assay[o] in accordance with the manufacturer's instructions and standardized at 1 to 5 mg/mL. Whole cell lysates were diluted 1:1 in 2X electrophoresis buffer (17% [vol/vol] glycerol, 8% [vol/vol] β-mercaptoethanol, 5% [wt/vol] SDS, 22% [vol/vol] 1M Tris-HCl [pH, 7.0], and 0.04% [wt/vol] bromophenol blue) and boiled at 90°C for 3 minutes. Proteins were resolved on a 10% SDS-poly-

acrylamide gel by means of electrophoresis and were electrotransferred to nitrocellulose membranes. Membranes were blocked in 5% (wt/vol) bovine serum albumin in Tris-buffered saline solution with 0.05% Tween 20[p] for 1 hour at room temperature (approx 22°C). Membranes were then probed with purified rabbit polyclonal anticleaved caspase-3[q] (Asp175) or purified rabbit polyclonal anti–β-actin.[q] After exposure to each primary antibody, blots were incubated with secondary antibodies (anti-rabbit IgG) conjugated with horseradish peroxidase at a final dilution of 1:1,000 for 1 hour at room temperature and bands were made visible with a chemiluminescence detection system,[r] in accordance with the manufacturer's instructions. Densitometric analysis was performed with image processing and analysis software.[s]

To investigate whether tulathromycin-treated neutrophils would be readily cleared by macrophages, myeloperoxidase activity was assessed in neutrophil-macrophage cocultures. For this portion of the in vitro experiment, neutrophils were treated by incubation with tulathromycin (0.2 mg/mL) or HBSS (control treatment) for 0.5 hours, washed with HBSS, and then cocultured with monocyte-derived porcine macrophages in a humidified chamber at 37°C and 5% CO_2 for 2 hours. Myeloperoxidase activity was measured in coculture supernatants and monolayers with a kinetic assay, as described elsewhere.[36] Photometric development of this assay was measured at 460 nm[k] at multiple intervals to yield enzyme activity data defined as the change in optical density with time (mU/min). Results are reported as fold change relative to activity in control cocultures; activity for control cultures was set to a value of 1.0.

Protocol for in vivo experiments

Following 7 days of acclimation, 3- to 4-week-old pigs were randomly assigned via weight ranking and drawing of group numbers to treatment groups in 2 in vivo experiments. In the first experiment involving experimentally induced *A pleuropneumoniae* infection, 3 treatment groups were used: one inoculated intratracheally with 2 mL of endotoxin-free PBS solution (vehicle [control treatment]; n = 12 pigs), one inoculated intratracheally with 1.5×10^7 CFUs of *A pleuropneumoniae* (13), and another injected IM with tulathromycin[l] (13) at the recommended dose (2.5 mg/kg) 0.25 hours prior to intratracheal inoculation with 1.5×10^7 CFUs of *A pleuropneumoniae*, as described elsewhere.[17]

In the second in vivo experiment designed to investigate zymosan-triggered pulmonary inflammation in the absence of bacterial stimuli, 3 groups were also used: one inoculated intratracheally with 2 mL of endotoxin-free PBS solution (vehicle [control group]; n = 7 pigs), one inoculated intratracheally with zymosan[t] (1 mg/kg; 7), and another injected IM with tulathromycin (2.5 mg/kg; 9) 0.25 hours prior to intratracheal inoculation with zymosan (1 mg/kg). Tulathromycin treatment in both in vivo experiments was intended to reflect metaphylactic administration, and the timing of this treatment has been validated.[29–31,34,35,37]

All pigs were given food and water ad libitum and housed at the animal care facilities of the University of Calgary. The environment was maintained at 21° to 23°C with 40% humidity, with the light cycle alternating between 12 hours of light and 12 hours of darkness. To offer additional microenvironment temperature regulation, pigs were provided with areas of solid flooring with black rubber mats, and 175-W red creep heat lamps were hung over a portion of the matted area to allow each pig to select an area within the pen where it felt comfortable. The solid flooring was of adequate area that all pigs could lie comfortably. Pigs were observed at least twice daily for assessment of sleeping and resting patterns to ensure they appeared comfortable with no signs of heat or cold stress during the study period. Pen washing was performed sparingly as needed to decrease the possibility that pigs might become chilled as a result of being wet. Care and experimental practices were conducted in accordance with standards of the Canadian Council on Animal Care[38] and approved by the University of Calgary Life and Environmental Science Animal Care Committee.

Preparation of bacterial isolates for inoculation—*Actinobacillus pleuropneumoniae* serotype 1,[u] which had been isolated from a pig that died of pleuropneumonia and preserved by freezing at –20°C, was used in this study as described elsewhere.[16] Bacteria were cultured from the frozen stock by streaking of thawed isolate on *Brucella* agar plates supplemented with 0.2% β-NAD and 5% horse serum and incubating overnight (10 to 14 hours) at 37°C in an atmosphere of 5% CO_2.[17] Thirty colonies were isolated and resuspended in 30 mL of PBS solution. Inocula were prepared by the addition of 1 mL of the bacterial suspension to 24 mL of prewarmed *Brucella* broth supplemented with 0.2% β-NAD and 5% horse serum. Inoculated broth was incubated for 3 to 4 hours in an orbital incubator at 150 revolutions/min, 37°C, and 5% CO_2 to generate mid to late logarithmic-phase bacterial suspensions. Experimental inocula were prepared by diluting *A pleuropneumoniae* suspension in endotoxin-free PBS solution to achieve a final concentration of 7.5×10^6 CFUs/mL

Intratracheal inoculation—Pigs were anesthetized by IM injection of azaperone (4 mg/kg) followed by administration of 5% isoflurane in O_2. Following induction of inhalation anesthesia, while pigs were still anesthetized, lidocaine spray was applied locally to the caudal aspect of the pharynx. A sterile catheter was inserted by use of an intubation tube, with the catheter tip positioned at the tracheal bifurcation. Pigs were challenged intratracheally with endotoxin-free PBS solution containing 1.5×10^7 CFUs of *A pleuropneumoniae* or zymosan (1 mg/kg), as described elsewhere.[17] Inoculum bacterial load was verified by spot plating on *Brucella* agar plates (data not shown).

Rectal temperatures were monitored prior to and throughout the experimental period to assess the health of all pigs involved.

BALF collection and analysis

Bronchoalveolar lavage was performed on pigs 3 or 24 hours after intratracheal challenge with *A pleuropneumoniae*. Following sedation and induction of anesthesia as described for the experimental inoculation procedure, pigs were endotracheally intubated and a sterile catheter was advanced through the tube to the tracheal bifurcation. Lavage was performed with 3 sequential washes of 10 mL of endotoxin-free HBSS, as described elsewhere.[17] Collected BALF samples were maintained on ice and processed immediately. Following BALF collection at 3 or 24 hours, pigs were euthanized while still anesthetized by intracardiac injection with sodium pentobarbital in accordance with the standards of the Canadian Council on Animal Care. Necropsies were immediately performed for gross morphological analysis and acquisition of lung tissue samples for histologic examination.

Cytocentrifuge samples were prepared by centrifugation[v] of BALF (100 µL/slide) at 113 X *g* for 10 minutes and staining[w] or fixation with freshly prepared 4% paraformaldehyde in PBS solution for cell identification and apoptosis detection, respectively. The degree of neutrophil infiltration in each sample was calculated as the percentage of neutrophils within the total leukocyte population detected within 3 microscope fields. Assessment of efferocytosis and phagocytosis was based on enumeration of macrophages containing apoptotic bodies

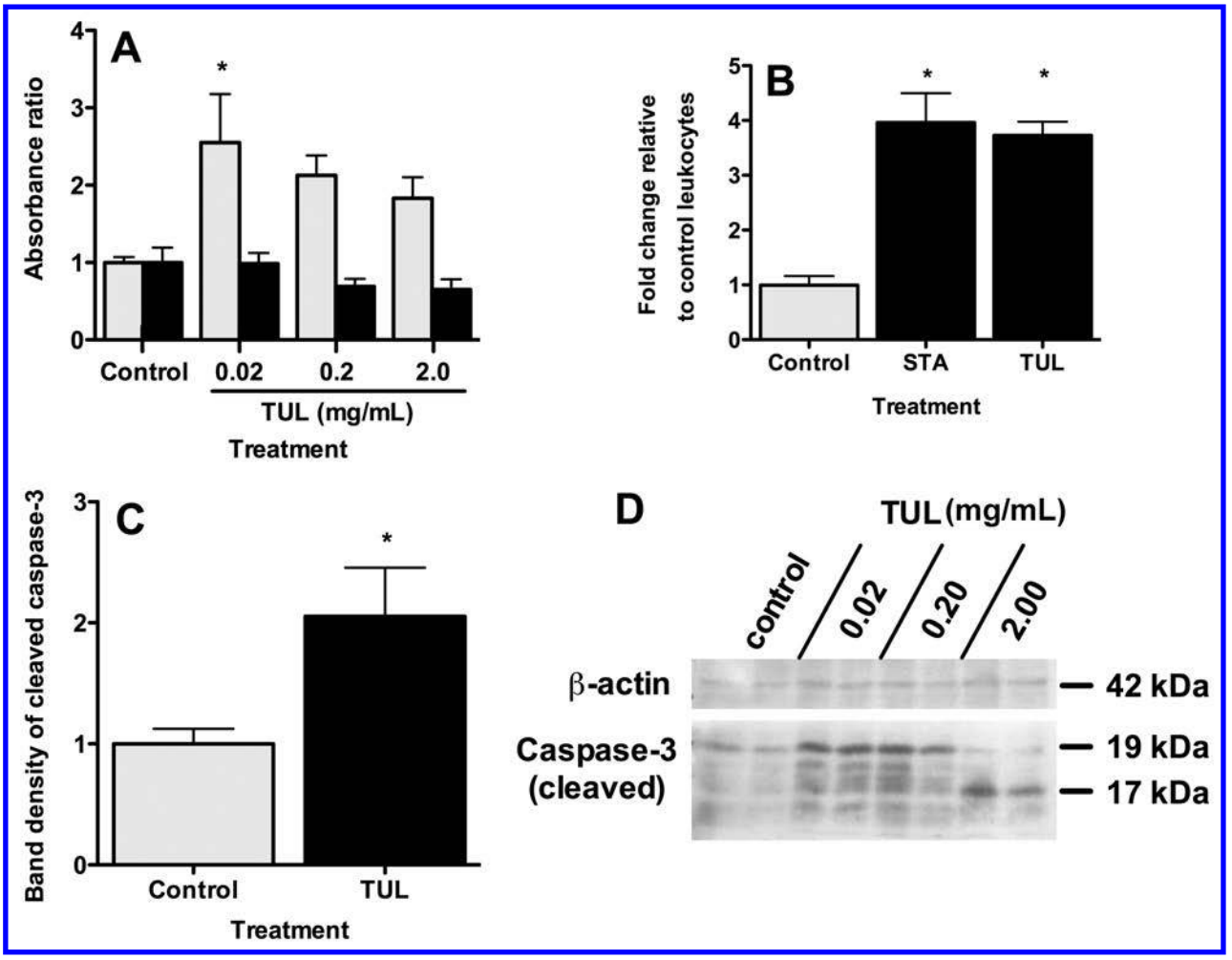

Figure 1—Effects of various concentrations of tulathromycin (TUL) on neutrophils isolated from the peripheral blood of 6 healthy male 8- to 30-week-old pigs. A—Results of an ELISA to identify apoptotic mono- and oligonucleosomes in neutrophils. Values were calculated as the ratio of light absorbance values for tulathromycin-treated cells versus values for control-treated cells (10% HI-FBS in HBSS; n = 4 repetitions/group) at 0.5 (gray bars) and 2 (black bars) hours of incubation. B—Results of TUNEL staining to identify apoptotic neutrophils following neutrophil incubation with TUL, staurosporine (STA), or 10% HI-FBS in HBSS (control). Values were calculated as the percentage of TUNEL-positive neutrophils within the microscopic field of view and are reported as a fold change relative to the degree of staining in control-treated cells (n = 3 repetitions/group). C—Results of densitometric analysis of western blot data for fragmentation of caspase-3 relative to β-actin in neutrophils. Values were calculated as the band density of cleaved caspase-3 (17 kDa) relative to that of β-actin and are reported as a ratio relative to the control value (n = 3 repetitions/group). D—Representative results of western blot analysis to detect caspase-3 fragmentation in neutrophils incubated with TUL at various concentrations. Value in panels A, B, C are reported as mean ± SEM. *Value is significantly ($P < 0.05$) different from the control value.

or particulate matter (zymosan). An aliquot of BALF from each sample was serially diluted and plated onto *Brucella* agar supplemented with 0.2% β-NAD and 5% horse serum and incubated overnight at 37°C. Colonies of *A pleuropneumoniae* on each plate were subsequently counted. The remaining BALF was centrifuged at 1,500 × *g* for 10 minutes. Aliquots of the BALF supernatants were snap frozen in liquid nitrogen and stored at −70°C for further analysis. Cell pellets were resuspended in HBSS, and cells counts were performed with a hemacytometer.[d] Remaining leukocytes were divided into aliquots of equal volumes for western blot analysis. Cell pellets were snap frozen in liquid nitrogen and stored at −70°C for further analysis.

Immunohistochemical analysis of lung tissue—Tissue samples obtained from cranial lung lobes during necropsy were fixed in formalin and embedded in paraffin prior to sectioning. Five-micrometer segments were prepared, mounted onto slides, and deparaffinized in preparation for H&E staining according to described methods.[39,40] Briefly, slides of tissue sections were stained in hematoxylin, rehydrated, and counterstained with eosin. Slides were examined by investigators who were not aware of their origin, and images were obtained at 400X magnification.[x]

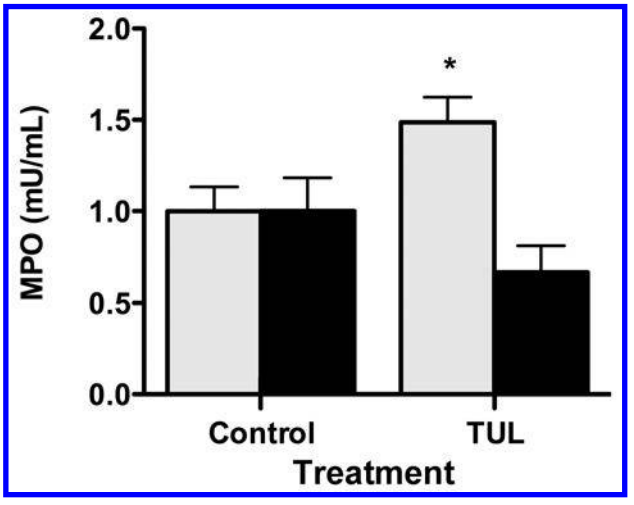

Figure 2—Mean ± SEM myeloperoxidase activity (MPO) in macrophage lysates (gray bars) and supernatants (black bars) after coculture of neutrophils pretreated with tulathromycin or HBSS (control) with monocyte-derived macrophages from the same pigs as in Figure 1 (n = 3 repetitions/group). *Value is significantly ($P < 0.05$) different from the corresponding control value. ***See*** Figure 1 for remainder of key.

Detection of LTB_4—To assess immunomodulatory effects of tulathromycin in vivo, LTB_4 concentrations in the neutrophil supernatants extracted from BALF samples were quantified by use of an ELISA kit[y] in accordance with the manufacturer's instruction. Concentrations of LTB_4 were determined at 405 nm by use of a microplate reader.[k] Reported specificity of the ELISA was 100% for LTB_4, 0.03% for 5(*S*)-hydroxyeicosatetraenoic acid, and < 0.01% for leukotrienes C_4, E_4, and D_4; it had a detection limit of 7 pg/mL

Detection of necrosis—Lactate dehydrogenase activity was quantified in BALF supernatants with a cytotoxicity detection kit,[z] in which colorimetric development was based on the reduction of tetrazolium to formazan, in accordance with the manufacturer's instructions. Degree of caspase fragmentation was measured in BALF neutrophils as described for the in vitro experiment.

Detection of efferocytosis—Efferocytosis was identified by direct microscopic examination of cytocentrifuged BALF preparations for macrophages containing 1 or more apoptotic neutrophils within phagocytic vacuoles. Findings are reported as the percentage of macrophages with efferocytosis among all macrophages.

Statistical analysis

All data were analyzed by means of 1-way ANOVA. Post hoc multiple comparisons were performed with the Tukey test. Values of $P < 0.05$ were considered significant. Summary data are reported as mean ± SEM (in some experiments, from multiple replicates of a minimum of 3 independent experiments).

Results

Effects of tulathromycin on leukocytes in vitro

After neutrophils from healthy 8- to 30-week-old pigs were incubated with tulathromycin (0.02 mg/mL) for 0.5 hours, the amount of apoptosis was significantly

Table 1—Mean ± SEM rectal temperatures, numbers of *Actinobacillus pleuropneumoniae* CFUs, and percentages of neutrophils recovered from BALF samples obtained from 3- to 4-week-old pigs 3 and 24 hours after intratracheal inoculation without (control) or with *A pleuropneumoniae* and pretreatment with or without tulathromycin (2.5 mg/kg, IM; n = 6 to 7 pigs/group).

	Control group		*A pleuropneumoniae*		*A pleuropneumoniae* and tulathromycin	
Variable	**3 h**	**24 h**	**3 h**	**24 h**	**3 h**	**24 h**
Bacteria (CFUs/mL)	1.58×10^2	7.75×10^2	8.65×10^{5}*	3.24×10^{7}†	7.82×10^{5}[a]	6.69×10^{4}‡
Neutrophils (%)	60.3 ± 6.6	43.4 ± 6.8	74.5 ± 5.2*	39.9 ± 10.2	74.8 ± 4.0*	46.4 ± 7.4
Rectal temperature (°C)	37.2 ± 0.3	38.2 ± 0.1	38.6 ± 0.6	39.4 ± 0.2	38.0 ± 0.3	38.1 ± 0.2

*Value is significantly ($P < 0.05$) different from the corresponding 3-hour control value. †Value is significantly ($P < 0.05$) different from the corresponding 24-hour control value. ‡Value is significantly ($P < 0.05$) different from the 24-hour value for untreated *A pleuropneumoniae*–inoculated pigs.

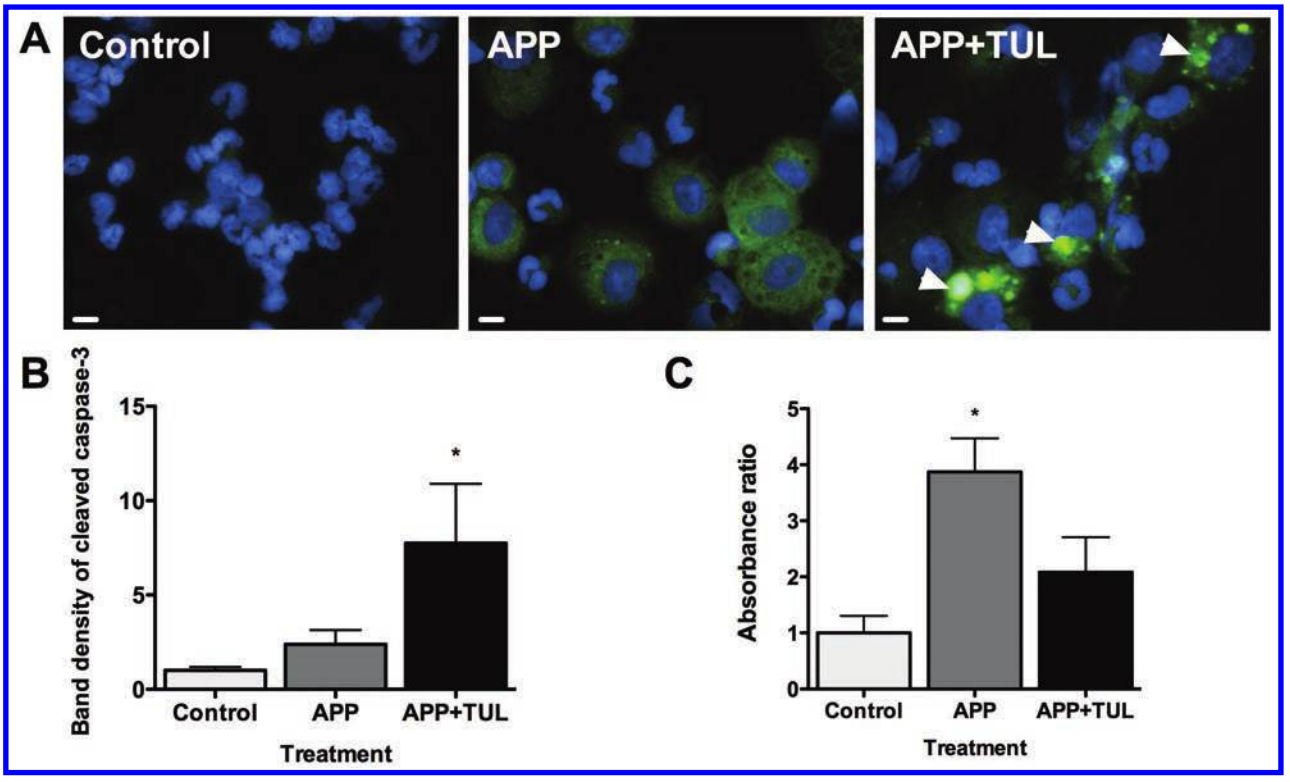

Figure 3—Results of quantification of apoptosis in leukocytes obtained from BALF of 3- to 4-week old pigs that were untreated and sham-inoculated (control; n = 12), untreated and intratracheally inoculated with *Actinobacillus pleuropneumoniae* (APP;13), or treated with tulathromycin (2.5 mg/kg, IM) 0.25 hours before intratracheal inoculation with *A pleuropneumoniae* (APP+TUL;13). A—Photomicrograph illustrating different degrees of TUNEL staining (green regions) in leukocytes. Blue regions indicate Hoechst nuclear counterstain. Notice the apoptotic leukocytes (arrowheads). Bars = 10 μm. B—Results of densitometric analysis of western blots of caspase-3 fragmentation in BALF samples obtained 24 hours after intratracheal inoculation. Values were calculated as the band density of cleaved caspase-3 (17 kDa) relative to that of β-actin and are reported as a ratio relative to the control value (n = 5 to 6 samples/group). C—Results of LDH quantification in BALF samples to assess the degree of cellular necrosis. Values were calculated as the ratio of light absorbance values relative to control values. Data in panels B and C are reported as mean ± SEM. *Value is significantly ($P < 0.05$) different from the control value. ***See*** Figure 1 for remainder of key.

greater than that in untreated control neutrophils **(Figure 1)**. After incubation for 2 hours, no effect of tulathromycin concentration (0.02 mg/mL to 2.0 mg/mL) on the degree of apoptosis was detected. The lowest concentration (0.02 mg/mL) was therefore selected for all subsequent testing. To corroborate these findings, the degree of DNA fragmentation within the neutrophils was examined, and results of TUNEL staining confirmed that incubation with tulathromycin significantly increased the number of TUNEL-positive neutrophils relative to the degree of staining in control neutrophils. Neutrophils incubated with staurosporine (1μM) as a positive control treatment also underwent apoptosis. Western blot analysis revealed that incubation with tulathromycin significantly increased fragmentation of caspase-3 in a concentration-dependent manner in neutrophils in vitro.

After incubation of neutrophils with HBSS or tulathromycin (0.02 mg/mL) for 2 hours and subsequent coculturing with macrophages from the same pig, myeloperoxidase activity was assessed in macrophage lysates and supernatant. In tulathromycin-treated cocultures, a significant increase was identified in intracellular myeloperoxidase activity relative to that in the control cocultures **(Figure 2)**.

Effects of tulathromycin in vivo

In 3- to 4-week-old pigs inoculated intratracheally with live *A pleuropneumoniae* (inoculated pigs), treatment with tulathromycin 0.25 hours before inoculation induced leukocyte apoptosis, inhibited the increase of LDH activity and LTB_4 concentration in BALF, and improved the histologic and macroscopic appearance of lung tissue following inoculation. Inoculated pigs had high numbers of bacteria and neutrophils in BALF samples collected 3 hours after inoculation **(Table 1)**. Inoculated pigs treated with tulathromycin had significantly fewer bacteria in their lungs at 24 hours after inoculation relative to the number of bacteria in the lungs of untreated inoculated pigs, in which bacterial loads remained significantly higher than those in untreated sham-inoculated control pigs. Neutrophil numbers in treated and untreated inoculated pigs did not differ significantly from those for control pigs at 24 hours after inoculation. Rectal temperatures did not differ significantly among treatment groups throughout the experimental infection period.

Densitometric analysis of BALF samples collected 3 hours after inoculation revealed no difference among the 3 treatment groups (data not shown). However, at

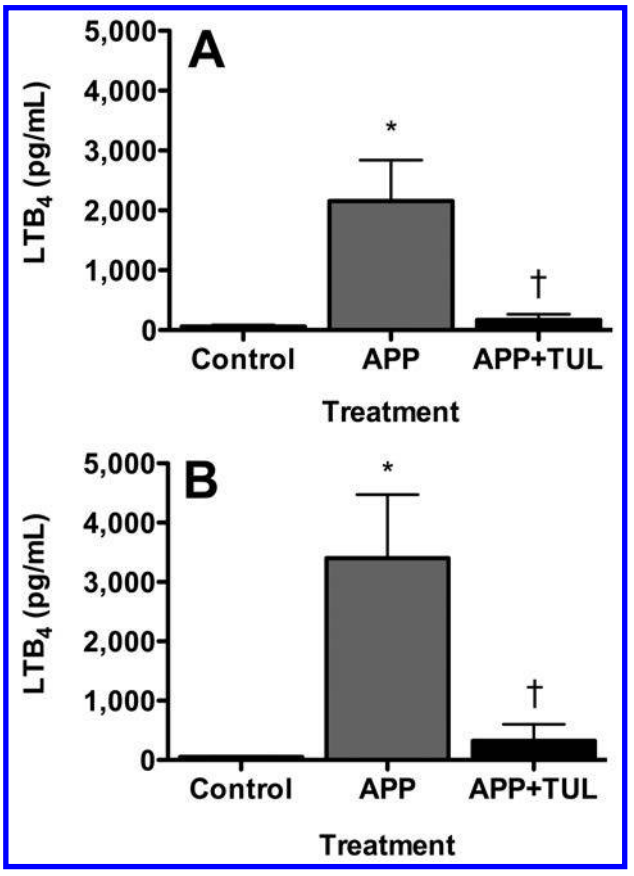

Figure 4—Mean ± SEM LTB_4 concentrations in BALF samples collected from the pigs in Figure 3 at 3 hours (n = 6 to 7/group; A) and 24 hours (6 to 7/group; B) after intratracheal inoculation with *A pleuropneumonia*. *Value is significantly ($P < 0.05$) different from the control value. †Value is significantly ($P < 0.05$) different from the value for untreated pigs intratracheally inoculated with *A pleuropneumoniae*. **See** Figures 1 and 3 for remainder of key.

24 hours after inoculation, an apparent increase in the numbers of TUNEL-positive leukocytes was detected within BALF samples **(Figure 3)**, and many of these apoptotic cells appeared to be contained within phagocytic vacuoles of macrophages. A significant increase in caspase-3 fragmentation was identified in samples collected 24 hours after inoculation. Consistent with these results, treatment with tulathromycin inhibited the increase in LDH and necrosis caused by *A pleuropneumonia* infection.

As early as 3 hours after inoculation, the BALF concentration of LTB_4 was significantly higher in untreated inoculated pigs than in control pigs. Treatment with tulathromycin inhibited this increase **(Figure 4)**. Notably, these differences were identified in the absence of a significant difference in bacterial loads between treated and untreated inoculated pigs (Table 1). By 24 hours after inoculation, the BALF LTB_4 concentration was likewise significantly greater in *A pleuropneumoniae*–inoculated pigs versus control pigs, and treatment with tulathromycin prevented this increase.

Necropsy and histologic evaluation of tissue samples obtained from the cranial lung lobes of pigs euthanized 3 hours after inoculation revealed that untreated inoculated pigs had evidence of edema and severe inflammatory infiltration and accumulation within the alveoli. On the other hand, control pigs evaluated at the same point had a distinct alveolar structure and clear alveolar spaces **(Figure 5)**. Conversely, treated *A pleuropneumoniae*–inoculated pigs had markedly less edema and inflammatory infiltrate within the alveolar spaces and an apparent reduction in inflammation relative to results for control pigs. Macroscopic examination of lung samples from untreated inoculated pigs euthanized 24 hours after inoculation revealed areas of apparent edema, tissue consolidation, necrotic foci, and fibrotic lesions that were absent in samples from control pigs at the same assessment point. In treated inoculated pigs at 24 hours after inoculation, these lesions were dramatically reduced in severity.

Effects of zymosan in vivo

The degree of pulmonary neutrophil infiltration in zymosan-inoculated pigs that had been euthanized and evaluated at 24 hours after inoculation was significantly greater than that in control pigs at the same evaluation point, and this increase was not altered by treatment with tulathromycin **(Table 2)**. Rectal temperatures did not differ significantly among treatment groups throughout the experimental infection period.

Fragmentation of caspase-3 in BALF leukocytes was evident as early as 3 hours after inoculation with zymosan, and treatment with tulathromycin significantly increased the degree of fragmentation relative to that in the control pigs **(Figure 6)**. However, this effect was no longer evident in pigs evaluated 24 hours after inoculation (data not shown).

Differential cell counts performed on BALF samples from pigs euthanized 3 hours after inoculation revealed that the degree of efferocytosis among macrophages was significantly greater in tulathromycin-treated inoculated pigs versus in control pigs, but not significantly different from the degree in untreated inoculated pigs **(Figure 7)**. At the same evaluation point, the percentage of phagocytosis-positive macrophages was similarly greater in treated and untreated, inoculated pigs than in control pigs. By 24 hours after inoculation, the degree of efferocytosis was similarly enhanced in treated and untreated, inoculated pigs (data not shown).

Analyses involving BALF samples obtained 3 hours after zymosan inoculation revealed a slight increase in LTB_4 concentrations in all pigs relative to expected values[17] **(Figure 8)**. By 24 hours after inoculation, LTB_4 concentrations were significantly higher in untreated zymosan-inoculated pigs than in control pigs, and treatment of inoculated pigs with tulathromycin significantly inhibited this increase.

Discussion

Medications that possess dual antimicrobial and

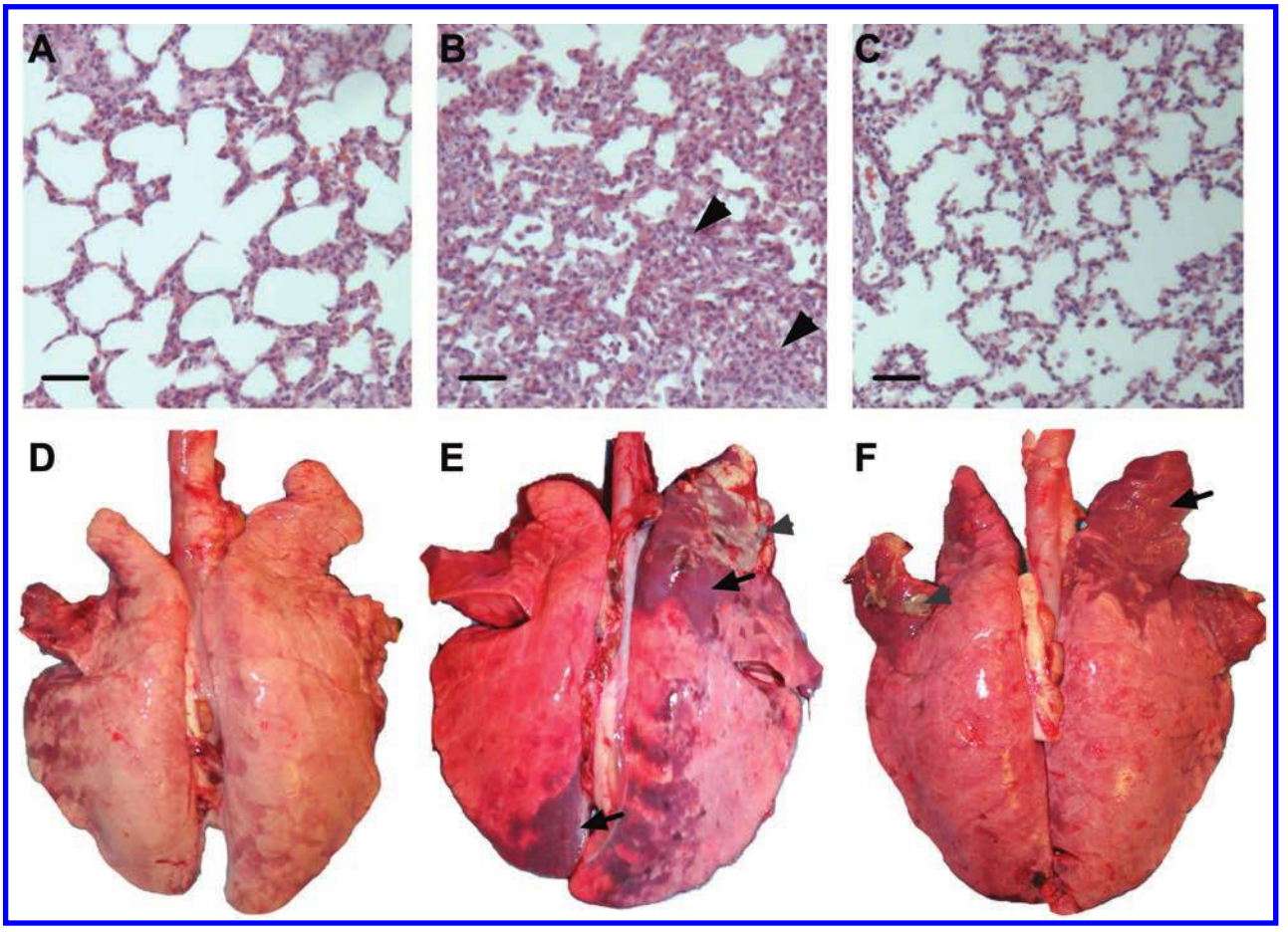

Figure 5—Representative photomicrographs (A–C) and photographs (D–F) of pulmonary tissue from untreated sham-inoculated pigs (A and D), untreated pigs intratracheally inoculated with *A pleuropneumoniae* (B and E), and pigs treated with tulathromycin (2.5 mg/kg, IM) 0.25 hours before intratracheal inoculation with *A pleuropneumoniae* (C and F). Photomicrographs show samples of cranial lobe lung tissue collected from pigs euthanized at 3 hours after inoculation. Notice the extensive neutrophil recruitment and a loss of apparent alveolar structure (arrowhead). H&E stain; bars = 100 µm. Photographs show evidence of severe pulmonary edema (arrows) and fibrotic lesions (arrowhead). ***See*** Figure 1 for remainder of key.

Table 2—Mean ± SEM rectal temperatures and percentages of neutrophils recovered from BALF samples from 3- to 4-week-old pigs at 3 and 24 hours after intratracheal inoculation without (control) or with zymosan (n = 3 to 5/group) and with or without pretreatment with tulathromycin (2.5 mg/kg, IM; 6 to 7 pigs/group).

	Control		Zymosan		Zymosan and tulathromycin	
Variable	**3 hours**	**24 hours**	**3 hours**	**24 hours**	**3 hours**	**24 hours**
Neutrophils (%)	61.0 ± 27.1	23.3 ± 3.1	95.5 ± 0.9	68.7 ± 5.6*	72.8 ± 13.6	68.2 ± 3.4*
Rectal temperature (°C)	37.5 ± 0.4	37.6 ± 0.5	37.3 ± 0.4	36.9 ± 0.2	37.4 ± 0.2	37.3 ± 0.2

*Value is significantly ($P < 0.05$) different from the corresponding 24-hour control value.

anti-inflammatory properties may be more efficacious than other medications for the treatment of infectious diseases in which the pathogenesis includes an inflammatory component.[17,41] Previous research has established that tulathromycin[31,34] and other macrolides[30,41] have immunomodulatory and anti-inflammatory characteristics. Results from the present study suggested that tulathromycin had similar cellular effects in 3- to 4-week-old pigs with experimentally induced *A pleuropneumoniae* infection or zymosan-induced pulmonary inflammation. Findings from the in vitro experiment involving leukocytes from 8- to 30-week old pigs indicated that tulathromycin promoted apoptosis in porcine neutrophils in a dose- and time-dependent manner, presumably as determined by the presence of mono- and oligonucleosomes. Supporting these findings were results of TUNEL staining and detection of caspase-3 fragmentation (the main caspase involved in

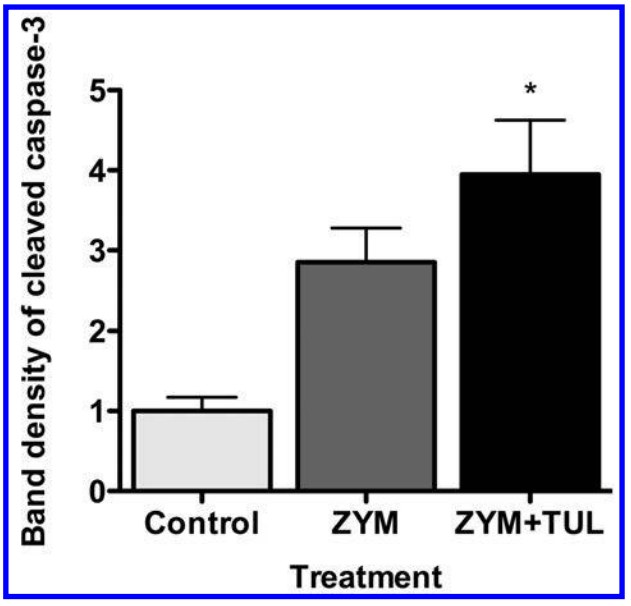

Figure 6—Mean ± SEM band density values from densitometric analysis of caspase-3 fragmentation in leukocytes isolated from BALF samples obtained from 3- to 4-week old pigs that were untreated and sham-inoculated (control; n = 7), untreated and intratracheally inoculated with zymosan (ZYM; 7), or treated with tulathromycin (2.5 mg/kg, IM) 0.25 hours before intratracheal inoculation with zymosan (ZYM+TUL; 9). Samples were obtained 3 hours after inoculation. ***See*** Figures 1 and 3 for remainder of key.

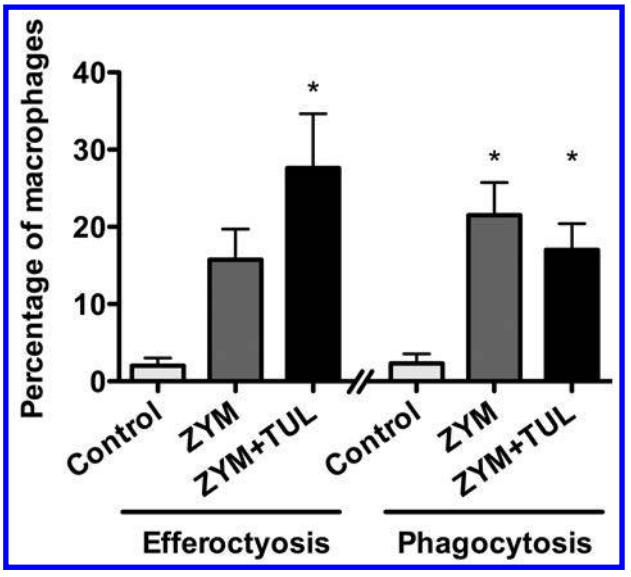

Figure 7—Mean ± SEM percentage of macrophages with evidence of efferocytosis and phagocytosis in BALF samples obtained from the same pigs as in Figure 6 at 3 hours after intratracheal inoculation with ZYM (n = 3 to 5 samples/group). A minimum of 3 microscopic fields were used to calculate the values. *Value is significantly ($P < 0.05$) different from the control value. ***See*** Figures 1 and 6 for remainder of key.

the execution phase of apoptosis[7]) in BALF samples obtained from the 3- to 4-week-old pigs. We also found that induction of apoptosis by tulathromycin facilitated cellular clearance by porcine macrophages, as

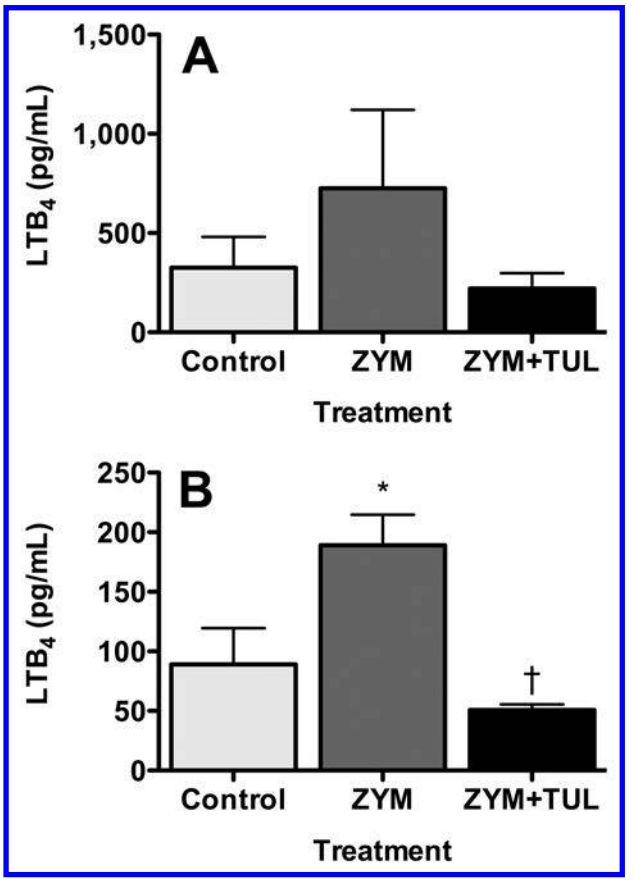

Figure 8—Mean ± SEM LTB_4 concentrations in BALF samples obtained from the same pigs as in Figure 6 at 3 hours (A) and 24 hours (B) after intratracheal inoculation with ZYM (n = 3 to 5 samples/group). *Value is significantly ($P < 0.05$) different from the control value. †Value is significantly ($P < 0.05$) different from the value for untreated ZYM-inoculated pigs. ***See*** Figures 1 and 6 for remainder of key.

indicated by intracellular myeloperoxidase activity in macrophage-neutrophil cocultures.

The aforementioned in vitro findings prompted us to investigate the effects of tulathromycin in an experimental model of *A pleuropneumoniae* infection. By 3 hours after intratracheal inoculation of pigs with *A pleuropneumoniae*, we identified a significant increase in the degree of fragmentation of caspase-3 within BALF leukocytes, which was accompanied by an apparent increase in the number of TUNEL-positive BALF leukocytes. Moreover, treatment with tulathromycin prevented the increase in tissue necrosis caused by infection. These effects were observed without a decrease in the total number of infiltrating neutrophils within the lungs throughout infection, suggesting that tulathromycin did not impact neutrophil recruitment, as reported elsewhere.[31] Furthermore, the findings indicated that tulathromycin inhibited the accumulation of proinflammatory LTB_4 within bronchoalveolar spaces of *A pleuropneumoniae*–inoculated pigs. Together, these findings suggested that tulathromycin had anti-inflammatory and proresolution benefits in *A pleuropneumoniae*–challenged lungs. This hypoth-

esis was also supported by the pulmonary histopathologic findings and gross pathological lesions, considering that treatment with tulathromycin reduced the severity of inflammatory infiltrate, edema, and lung lesions relative to that in untreated but similarly inoculated pigs, thereby suggesting an improved prognosis after inoculation. The slight increase evident in BALF LTB_4 concentrations of all pigs relative to expected values[17] was likely a result of the lavage procedure.

Results of the *A pleuropneumonia* infection experiment prompted us to evaluate anti-inflammatory and proresolution properties of tulathromycin independent of its antimicrobial effects with an in vivo model of pulmonary inflammation (caused by zymosan) in the absence of live bacteria. Similar to results obtained for the *A pleuropneumonia* infection experiment, tulathromycin had proapoptotic properties early in the nonbacterial, zymosan-induced inflammation experiment, without impacting neutrophil recruitment to the bronchoalveolar spaces. Tulathromycin also appeared to promote the preferential efferocytosis of apoptotic leukocytes at the same time point, suggesting enhanced nonphlogistic clearance of the dying cells. Perhaps most striking, however, was the observation that tulathromycin inhibited LTB_4 production in zymosan-challenged lungs, a distinct anti-inflammatory effect in the absence of bacterial stimuli. These findings suggested that tulathromycin had immunomodulatory properties independent of its antimicrobial effects and that these properties may be particularly advantageous in the treatment of infectious inflammatory diseases.

Severe tissue damage and rapid pathogenesis of *A pleuropneumoniae*–induced pneumonia is in part a result of local cellular necrosis and self-perpetuating recruitment of neutrophils to the site of infection. Investigators in a previous study[1] determined the efficacy of drugs that target neutrophil activation in delaying lesion development associated with *A pleuropneumoniae* infection. Our findings provided evidence of the anti-inflammatory and immunomodulatory properties of tulathromycin in a clinically relevant model of bacterial pneumonia within a target species. Within the lungs of pigs, *A pleuropneumoniae* exotoxins (I, II, and III) cause lysis of infiltrating neutrophils and macrophages as well as alveolar epithelial and endothelial cells.[42,43] Cell lysis causes the release of cytotoxic cellular contents into the extracellular milieu, which damages surrounding cells and promotes the secretion of proinflammatory mediators. These mediators in turn amplify leukocyte recruitment and exacerbate inflammatory injury.[12] Findings of the present study suggested that tulathromycin was able to interrupt the inflammatory cascade by promoting controlled apoptosis of leukocytes within the lungs and that this proapoptotic effect was associated with an improvement in pulmonary histologic findings and a decrease in lesion development in infected pigs. Correspondingly, tulathromycin inhibited the increase in tissue necrosis caused by infection. In vitro evidence further supported the in vivo findings and was consistent with previous reports[31,34] of the mechanisms through which tulathromycin exerts proapoptotic effects. Similarly, other macrolides including azithromycin and tilmicosin appear to have a propensity for the induction of neutrophil apoptosis.[28–30,38,44,45] Moreover, effects in the present study occurred without impacting the degree of neutrophil recruitment to the lungs, similar to findings for models of pneumonia in cattle[31] and consistent with reports[17,29,43] for other macrolides.

Precise mechanisms through which tulathromycin exerts its anti-inflammatory and immunomodulatory effects are incompletely understood, but it appears to signal through caspase-3.[31,34] Controlled apoptosis of infiltrating leukocytes is an essential component in the resolution of inflammation following infection.[7] Additional investigation is warranted to determine the apoptotic pathway through which tulathromycin acts and whether it acts through similar mechanisms in porcine cells as it does in bovine cells.[31,34]

During severe inflammation, infiltrating neutrophils release LTB_4, a potent neutrophil chemoattractant derived from arachidonic acid that contributes considerably to the self-amplifying recruitment of neutrophils to the site of inflammation[3,4,17] and the development of pulmonary lesions in *A pleuropneumoniae* infection.[1] For the resolution of inflammation to occur following infection, proinflammatory signals must first be dampened to curtail leukocyte recruitment. Other macrolides can inhibit the production of proinflammatory cytokines[41,46] and suppress NF-κB activation.[47–49] Results of the present study indicated that treatment with tulathromycin inhibited LTB_4 production in infected lungs as well as in inflamed zymosan-challenged lungs in pigs. These findings are consistent with those for an experimental model of *Mannheimia haemolytica*–induced pneumonia in cattle,[31] and similar observations have been reported for tilmicosin, another macrolide that also promotes apoptosis.[39] Notably, our findings were observed in the presence of a bacterial challenge as well as in a nonbacterial inflammatory challenge, suggesting that the immunomodulatory and anti-inflammatory properties of tulathromycin may be independent of its antimicrobial properties. Additional research is warranted to investigate whether these anti-inflammatory effects of tulathromycin were independent of its proapoptotic properties. Nevertheless, the inhibition of proinflammatory mediator production may contribute considerably to the promotion of inflammation resolution following infection.

Cellular death by apoptosis is a critical step in the resolution of inflammation following infection because it facilitates nonphlogistic removal of infiltrating cells from the site of injury.[7,50,51] The process of apoptosis is characterized by several morphological changes including cellular shrinkage, chromatin condensation, and DNA fragmentation.[52,53] Cells under

going apoptosis also lose some of their functional characteristics and become hyporesponsive to external stimuli, which helps facilitate their nonphlogistic removal from the site of inflammation.[8,54] In addition, apoptotic cells express signals necessary to trigger their phagocytic uptake by macrophages in a process known as efferocytosis.[11,55] For example, apoptotic cells express phosphatidylserine on the outer surface of the cell membrane to signal their death to adjacent phagocytes.[56] Receptors associated with efferocytosis of apoptotic cells include a phosphatidylserine receptor, CD36, CD14, and CD68.[57,58] Recognition of apoptotic cells by macrophages facilitates clearance of these cells from the tissues, which prevents damage from postapoptotic necrosis[59] and can also trigger an anti-inflammatory phenotype in efferocytic macrophages that promotes the release of anti-inflammatory mediators such as transforming growth factor-β and IL-10.[10,50] Together, these processes help resolve inflammation and restore tissue homeostasis.

Findings in the present study suggested that tulathromycin promoted apoptosis within infected and inflamed lungs in pigs and that tulathromycin also enhanced efferocytosis of apoptotic cells. The results were consistent with reported results involving bovine cells[35] and were corroborated by our in vitro findings, which suggested that neutrophils that underwent tulathromycin-induced apoptosis were phagocytosed by macrophages with greater avidity than were control cells. Collectively, the findings indicated that tulathromycin had immunomodulatory properties in leukocytes that conferred anti-inflammatory and proresolution benefits in the context of bacterial pneumonia as well as pulmonary inflammation in pigs. These benefits may explain at least in part the efficacy of tulathromycin in the treatment of respiratory disease in pigs caused by *A pleuropneumoniae*.[60] Additional research is warranted to investigate the effects, if any, that tulathromycin may have on the signaling of proresolution lipid mediators.

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Footnotes

a. Vacutainer, Becton-Dickinson, Franklin Lakes, NJ.
b. Sigma-Aldrich, Oakville, ON, Canada.
c. PAA Laboratories Inc, Dartmouth, Mass.
d. VWR Scientific, Edmonton, AB, Canada.
e. Gibco, Grand Island, NY.
f. Diff-Quik stain set, Siemens Healthcare Diagnostics, Tarrytown, NY.
g. CytoSpin 4 cytocentrifuge, Thermo Scientific, Burlington, ON, Canada.
h. Costar, Cambridge, Mass.
i. Draxxin, Zoetis, Kalamazoo, Mich.
j. Cell Death ELISA, Roche Diagnostics, Laval, QC, Canada.
k. SpectraMAX M2e microplate reader, Molecular Devices, Menlo Park, Calif.
l. Sigma-Aldrich, Oakville, ON, Canada.
m. Cell Death TUNEL kit, Roche Diagnostics, Laval, QC, Canada.
n. Cell lysis buffer solution, Roche Diagnostics, Laval, QC, Canada.
o. Bio-Rad Laboratories, Mississauga, ON, Canada.
p. TWEEN 20, Roche Diagnostics, Laval, QC, Canada.
q. Cell Signaling, Beverly, Mass.
r. Amersham ECL Western Blotting Detection Reagent, GE Healthcare Life Sciences, Pittsburgh, Pa.
s. ImageJ, version 1.48, National Institutes of Health, Bethesda, Md. Available at: rsbweb.nih.gov/ij/index.html. Accessed Jun 16, 2014.
t. Sigma-Aldrich, Oakville, ON, Canada.
u. Provided by Dr. M. Gottschalk, University of Montreal, St-Hyancinth, QC, Canada.
v. CytoSpin 4 cytocentrifuge, Thermo Scientific, Burlington, ON, Canada.
w. Diff-Quik stain set, Siemens Healthcare Diagnostics, Tarrytown, NY.
x. Retiga 2000X with Q Capture Suite software, Q Imaging, Surrey, BC, Canada
y. LTB4 ELISA Cayman Chemical Co, Ann Arbor, Mich.
z. Cytotoxicity detection kit (LDH), Roche Diagnostics, Laval, QC, Canada.

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