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Short communication

Effects of priming with cytokines on intracellular survival and replication of *Rhodococcus equi* in equine macrophages

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ABSTRACT

Rhodococcus equi is a common cause of pneumonia in foals and an opportunistic pathogen in immunosuppressed people. The ability of *R. equi* to survive and replicate in macrophages is the basis of its pathogenicity. Limited knowledge about the role of cytokines in host defense against *R. equi* comes from studies in mice and the role of cytokines in intracellular survival of *R. equi* in equine macrophages is unknown. The objectives of this study were to determine the effect of priming with interferon (IFN)-γ, interleukin (IL)-1β, IL-4, IL-6, IL-10, or tumor necrosis factor (TNF)-α at various concentrations on intracellular survival of virulent *R. equi* in equine monocyte-derived macrophages (MDM), and to determine the effects of various combinations of the same cytokines on intracellular survival of *R. equi*. MDM from 10 adult horses were primed with recombinant equine cytokines at doubling concentrations ranging from 25 to 200 ng/mL prior to infection with virulent *R. equi*. Priming with IFN-γ, TNF-α, or IL-6 significantly decreased intracellular replication of *R. equi* compared to unprimed monolayers. In contrast, priming with IL-10 or IL-1β significantly increased intracellular replication of *R. equi*. Pairwise combinations of the cytokines listed above did not results in synergism or antagonism. This study demonstrated that IFN-γ, TNF-α, or IL-6 improved equine MDM function against *R. equi* whereas IL-1β or IL-10 were detrimental.

1. Introduction

Rhodococcus equi, a Gram-positive facultative intracellular pathogen that primarily infects macrophages, is one of the most important causes of disease in foals between 3 weeks and 5 months of age. *R. equi* has also emerged as a common opportunistic pathogen in immunosuppressed people, especially those infected with the human immunodeficiency virus or receiving chemotherapy [1,9,26]. Infection in either species is most commonly characterized by life-threatening pyogranulomatous cavitating pneumonia although systemic infections occur occasionally [23,26].

The ability of *R. equi* to survive and replicate in macrophages is at the basis of its pathogenicity. The ability of *R. equi* to survive and replicate in macrophages is dependent on expression of VapA encoded in the pathogenicity island of the virulence plasmid [16]. Multiple studies have confirmed that survival and replication of *R. equi* in macrophages concurs with virulence studies in both mice and foals [3,12,16]. Most of what is known about immunity to *R. equi* comes from studies in mice, in which $CD4^+$ T helper 1 (Th1) lymphocytes producing IFN-γ are essential for protection whereas a Th2 response is detrimental and leads to development of pulmonary granulomas [18]. Direct assumptions regarding a protective immune response in horses based on extrapolations from the mouse model could be misleading given that immunocompetent mice are resistant to infections caused by *R. equi*. The role of cytokines in intracellular survival of *R. equi* in equine macrophages is unknown. Identification of the function of these cytokines is important for developing new control strategies.

The objectives of this study were to determine the effect of priming with interferon (IFN)-γ, interleukin (IL)-1β, IL-4, IL-6, IL-10, or tumor necrosis factor (TNF)-α at various concentrations on intracellular survival of virulent *R. equi* in equine monocyte-derived macrophages (MDM), and to determine the effects of various pairwise combinations of the same cytokines on intracellular survival of *R. equi*.

2. Materials and methods

2.1. Animals and sample collection

Twenty adult horses between 3 and 18 years of age were used. Ten horses were used to test the optimal concentration of each cytokine and 10 different horses were used to assess the effects of pairwise concentrations of cytokines. Animals were considered healthy based on

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physical examination and complete blood count. Blood (1.5 L per horse) was collected by jugular venipuncture in glass bottles containing EDTA as the anticoagulant. The study was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

2.2. Isolation of monocyte-derived macrophages

Mononuclear cells were harvested from blood by density gradient centrifugation (Ficoll-Paque, Amersham Biosciences, Pittsburgh, PA), washed 3 times with phosphate buffered saline (PBS), and counted by use of an automated cell counter (Cellometer Auto T4, Nexelom Bioscience, Lawrence, MA). MDM were obtained exactly as described previously [2]. Blood mononuclear cells were suspended at a concentration of 4×10^6 cells/mL in Minimum Essential Medium-alpha (MEMα) containing 10% horse serum (HS), penicillin G (100 U/mL), streptomycin (80 μg/mL), and recombinant equine IFN-γ, IL-1β, IL-4, IL-6, IL-10, or TNF-α (R & D Systems, Minneapolis). In the first experiment, recombinant cytokines were used at concentrations of 25, 50, 100, and 200 ng/mL. The wide range of concentrations was selected based on studies in murine macrophages evaluating the effect of these cytokines on intracellular growth of various bacterial pathogens [4,10,21,22]. In a preliminary experiment using 6 horses, MDM exposed to IFN-γ and IL-4 at the concentrations listed above were incubated both in the presence or absence of lipopolysaccharide (LPS; 055:B5) at a concentration of 100 pg/mL. Based on results of this preliminary study, LPS was not used for the other cytokines or for the second experiment. In the second experiment, pairwise combinations of all the cytokines listed above were evaluated at a concentration of 50 ng/mL based on the results of the first experiment. For all experiments, cells not exposed to cytokines were used as negative controls. The cells were incubated for 18 h at 37 °C in 6% CO_2. Non-adherent and loosely adherent cells were removed and the remaining MDM were harvested, pelleted, and suspended at a concentration of 1×10^6 macrophage/mL in MEMα containing 10% HS.

2.3. Infection of cells for intracellular survival and replication assay

The MDM suspension (0.5 mL) was added to each well of a 24 well plate (Nunc, ThermoFisher Scientific, Rochester NY). After a 3-h incubation, media was removed and MDM were infected with virulent *R. equi* (ATCC #33,701, Rockville, MD) at a ratio of 1 bacterium per macrophage. The cells were incubated for 40 min to allow phagocytosis. Noninfected macrophage monolayers cultured under the same conditions were used as negative controls. Media containing *R. equi* was removed carefully and each well was washed 3 times with PBS. Three wells of infected cells were lysed and used to determine initial bacterial load (Time 0). All other wells were incubated in MEMα supplemented with 10% HS, recombinant cytokines at the appropriate concentration (see above), and 8 μg of amikacin sulfate per mL to kill remaining extracellular *R. equi* and to prevent extracellular growth with continuous re-infection of macrophages [14]. At time 0 (immediately post-infection) and 48 h post-infection, supernatants were removed and monolayers were washed 3 times with PBS. The number of colony forming units (CFU) per well was determined as previously described [2]. The mean of triplicate wells was used for data analysis. The data were displayed as $\log_{10}$ of the mean fold-change in CFU over a 48 h period ($\log_{10}$ [CFU at 48 h/CFU at Time 0]).

2.4. Statistical analysis

Normality of the data was assessed based on examination of histograms and normal quantile plots of residuals. Constant variance of the data was assessed by plotting residuals against predicted values. Data were log-transform to meet the distributional assumptions. Data were analyzed using linear mixed-effects modeling with horse modeled as a random effect and cytokine concentration (experiment 1), presence of LPS (preliminary study for IFN-γ and IL-4 only), or cytokine combination (experiment 2) as fixed nominal effects. When applicable, relevant 2-way interactions were also included in the model. Model fit was assessed using Akaike's information criterion values. For the purpose of this study, synergism between 2 cytokines was defined as a statistically significant decrease in intracellular replication of *R. equi* relative to that obtained with each cytokine alone. Conversely, antagonism between 2 cytokines was defined as a statistically significant increase in intracellular replication of *R. equi* relative to that obtained with each cytokine alone. When indicated based on the F-test of overall significance, multiple pairwise comparisons to the negative control cells or to relevant cytokines were performed using the method of Holm–Sidak to control for family-wise type 1 error rates. For all analyses, significance was set at $P < .05$.

3. Results

Classical macrophage activation has been historically defined as IFN-γ priming followed by LPS exposure as a triggering signal [24]. Therefore, the effect of LPS as a triggering signal was evaluated in a preliminary experiment. Intracellular replication of *R. equi* in MDM was significantly (P = .005) increased in cells exposed to LPS compared to that of cells not exposed to LPS in both the IFN-γ (P = .025) and IL-4 (P = .005) experiments (Fig. 1A). There were no significant interactions between presence of LPS and concentration of IFN-γ (P = .290) or IL-4 (P = .291) indicating that the effects of these 2 cytokines on intracellular replication of *R. equi* did not depend on the presence of LPS. Therefore, data generated in the absence of LPS were used for subsequent experiments. Priming of MDM with recombinant IFN-γ significantly decreased intracellular replication of *R. equi* compared to unprimed monolayers regardless of the concentration used (Fig. 1B). IL-6 and TNF-α resulted in a significant decrease in *R. equi* CFUs, but only at a single concentration. In contrast, priming with recombinant IL-10 or IL-1β significantly increased intracellular replication of *R. equi* regardless of the concentration (Fig. 1B). Based on these results, a concentration of 50 ng/mL was selected for the pairwise combination of cytokines. Pairwise combinations of the cytokines listed above did not results in synergism or antagonism (Fig. 2). However, combination with IFN-γ prevented the ability of IL-10 to increase intracellular replication of *R. equi* whereas combination with TNF-α or IL-6 did not prevent the negative effect of IL-10 (Fig. 2).

4. Discussion

Although multiple studies have investigated cytokine induction after experimental infection of equine phagocytic cells with *R. equi*, the effects of cytokines on intracellular survival of *R. equi* in equine macrophages could not be investigated due to the lack of immunological reagents for horses. The recent availability of biologically active recombinant equine cytokines has made it possible to assess the role of various cytokines on equine macrophage activation and function. This study demonstrates that priming of equine MDM with IFN-γ, TNF-α, or IL-6 decreases intracellular replication and survival of virulent *R. equi*, whereas IL-10 or IL-1β have the opposite effect. Because the outcome of macrophage infection with *R. equi* and the outcome in the host are correlated [12], infection of macrophages with *R. equi* represents a simple model to study modulation of host responses in horses without the need for creating disease in foals.

The ability of IFN-γ and TNF-α to enhance equine MDM function against *R. equi* is not unexpected given their roles in host defense against other Gram-positive facultative intracellular bacterial pathogens [15]. The results of this study are also consistent with those of experimental infection of mice with virulent *R. equi* in which depletion of IFN-γ or TNF-α results in failure to clear the pathogen [17,19]. In addition, IFN-γ has been shown to decrease intracellular survival of virulent *R. equi* in murine macrophages [5,25]. The role of IL-6 during