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Research Article

Serum Derived Transfer Factor Stimulates the Innate Immune System to Improve Survival Traits in High Risk Pathogen Scenarios

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Strategy, Management and Health Policy				
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ABSTRACT Transfer Factors (TFs) are low molecular weight (<5,000 daltons) biological response mediators. In the present study, a serum derived TF improved the ability of the recipient animal to survive high-risk infectious challenges (salmonellosis and canine parvoviral enteritis (CPV)) by altering the host's cytokine response profile. Mice mortally challenged with 5,000 colony-forming units of *Salmonella* experienced a group mortality of 73% while mice treated with a single 5 mg dose of the TF demonstrated a significant decrease in morbidity (7%, $p \leq 0.01$). The splenic bacterial load in untreated mice was over 10,000 times higher than that in the TF treated mice. Twenty-four hours post-administration, the treated murine population expressed a rapid temporal increase in serum IL-6 (26-fold) and INF-γ (77-fold) concentrations. IL-6 can act as a critical signal regulating action against bacterial pathogens. A comparative double-blind study performed using dogs confirmed to be undergoing a canine parvovirus challenge showed that when conventional supportive therapy was supplemented with a single 5 mg TF dose there was a reduction ($p \leq 0.01$) in group mortality (68% of the TF treated group survived versus 32% of the placebo group), an observation consistent with the observed increase in INF-γ, a cytokine associated with promoting antiviral activity. Drug Dev Res 00 : 000000, 2017. © 2017 Wiley Periodicals, Inc.

Key words: biological response modifier; immunotherapy; antiviral

INTRODUCTION

Transfer Factors (TFs) are low molecular weight (<5,000 daltons) leukocyte dialysates that passively transfer cell mediated immunity to a non-immune recipient [Kirkpatrick, 2000]. They have been observed to provide prophylactic and therapeutic control benefit for bacterial tuberculosis [Fabre et al., 2004], leprosy [Bullock et al., 1972], viral challenges (*herpes simplex* [Estrada et al., 1995] and *varicella zoster* [Steele et al., 1980]), and parasite infections (leishmaniasis [Delgado et al., 1981] and toxoplasmosis [Chinchilla and Frenkel,

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1984]). TFs can also promote an enhanced vaccine response to porcine parvovirus [Wang et al., 2012].

In the present study, the beneficial effects of an unconventional "serum"- derived TF were shown in animals under duress from either a bacterial or viral presence. From these findings we hypothesize that biological response mediation through TFs may improve survival outcomes when animals undergo a pathogenic crisis.

MATERIALS AND METHODS

Transfer Factor (TF): Preparation and Formulation

Transfer Factor (TF) was prepared by filtering normal sterile caprine serum (Colorado Serum Company, Denver, CO) to obtain molecular weight components of less than 10,000 daltons. The isolate was then lyophilized and stored at −20°C and rehydrated in physiologic saline as a 10 mg/ml formulation for canine studies and 25 mg/ml for murine studies.

Mouse Studies: Murine Salmonellosis Experimental Design (Salmonella Challenge Assay)

Four to six-week-old female Swiss Webster mice (Envigo, Prattville, AL), weighing 18–24 g, were housed up to five per cage and a minimum of six cages were used per treatment group in a randomized block design. Mice comprising the control and treated populations were injected ip with 0.20 ml of *S. enterica* ser. Typhimurium modified with the pAK1-lux plasmid construct ($\sim 5 \times 10^3$ CFU/mouse) in order to permit the live imaging of *Salmonella* proliferation via bioluminescence. TF treated mice received 5 mg TF while control mice received a saline placebo, each as a 0.20 ml subcutaneous injection 24 hours before challenge. Mice were monitored three times daily for one week; animals found moribund were euthanized by exposure to carbon dioxide. Three mice from each group were randomly selected to image on days 2, 3, and 4 post challenge. Spleens were harvested from these animals for bioluminescent imaging and bacterial quantitation (CFU/g). Serum was also collected from three randomly selected mice at 0, 3, 10, and 12 hours post TF administration.

This study was conducted in accordance with the NRC *Guide for the Care and Use of Laboratory Animals* and was approved by the Mississippi State University Institutional and Animal Care and Use Committee.

Imaging

Mice were restrained with 1.5–3.0% isoflurane (Piramal, India) via nose cone and maintained on a 37°C heated platform to ensure stability while photonic emissions were recorded for 5 minutes with an IVIS 100 biophotonic imaging system (Xenogen Corporation, Alameda, CA) according to manufacturer's instructions [Ryan et al., 2005]. Harvested tissues (spleens) were imaged in an analogous manner.

Cytokine Assay

Serum derived from naïve and TF treated (5mg sc 24 hours prior to harvesting blood) mice was analyzed for IL-6 and INF-γ by using the multiplex proinflammatory cytokine assay (Pierce Biotechnology, Rockford, IL). Serum was also analyzed via mass spectral analyses to ascertain changes within the proteome (serum amyloid A protein and interferon inducible protein 1 (Irgm1)) with respect to time.

Viral Challenge Studies

Selection criteria for CPV survival

Dogs, including both males and females of different breeds, between 5 and 16 weeks of age (originating from two local pet stores) were included in the study if diagnosed with canine parvoviral enteritis by clinical evaluation and fecal ELISA analysis (IDEXX, Westbrook, ME).

CPV supportive therapy

All animals were placed in an isolation ward (Collierville, TN) and provided cefazolin (22 mg/kg iv every 8 hours; Pfizer, New York, NY) to combat bacterial infection, metoclopramide (0.5 mg/kg sc every 8 hours; Hospira, Inc., Lake Forest, IL) to normalize gastrointestinal peristalsis and reduce vomiting, and 5% dextrose lactated Ringers solution (Hospira, Inc., Lake Forest, IL) with 20 mEq KCl/L (Hospira, Inc., Lake Forest, IL), a physiologic electrolyte solution used to restore fluid and electrolyte balance. This represented an accepted standard of care for CPV at the time of the study [Goddard and Leisewitz, 2010].

CPV experimental design

Upon admission to the study, the dogs were randomly assigned to one of two treatment groups (A: TF and B: Placebo). Supportive care was initiated and 0.5 ml representing 5 mg of test article (TF) or a placebo of physiological saline was administered as a single subcutaneous injection. The veterinarian and staff personnel were blinded as to the identity of the

treatment groups. Animal survival was the primary parameter of therapeutic success. Severity of the disease upon presentation was appraised by assessing the following parameters: diarrhea (0–3) where 0 = none, 1 = mild (soft-formed stool), 2 = moderate (runny stool), and 3 = severe (loose stool with excessive mucous/blood); apathy (0–2) where 0 = none, 1 = mild to moderate, and 2 = severe depression/comatose; vomiting (0 = no, 1 = yes), fever (0 = no, 1 = yes), and overall assessment of presenting condition (0–3) where 0 = no symptoms, 1 = mild symptoms, 2 = moderate symptoms, and 3 = severe symptoms. Other existing conditions (i.e., secondary infections or concurrent infections) were noted.

Cell culture and medium

S. enterica ser. Typhimurium (ATCC # 14028; Manassas, VA) transformed with plasmid pAK1-lux were spread on Brilliant Green Agar (BG) supplemented with Ampicillin Sodium Salt, (2 μg/ml; Sigma-Aldrich, Inc., St. Louis, MO) for determination of colony forming units [Moulton, 2009]. Inoculum cultures were prepared in Brain Heart Infusion Broth at 37°C for 24 hours on an orbital shaker. Serial dilutions were conducted in sterile Dulbecco phosphate buffered saline (dPBS).

Canine fibroblast A-72 cells from the American Type Culture Collection (ATCC CRL-1542) were cultured in Eagles minimal essential medium supplemented with 5–10% (v/v) heat inactivated Fetal Bovine Serum, 100 units/ml penicillin, 10 μg/ml gentamicin, and 2.5 μg/ml fungizone. Cultures were maintained as monolayers in tissue culture labware at 36–38°C in a humidified atmosphere of 5–7% CO_2.

Canine parvovirus

Canine parvovirus (VR-2017) was obtained from the ATCC and cryogenically stored at −70°C. The virus was propagated in canine fibroblast A-72 cells in disposable tissue culture labware as described above.

Virucidal and cytotoxic experimental design

The virucidal and antiviral interactions between TF and CPV were assessed by ViroMed Biosafety Laboratories (St. Paul, MN). All tests were replicated ($n \geq 3$). In the virucidal assay, CPV was incubated with log dilutions of TF (1 pg/ml to 1 mg/ml) for four hours, and then added to wells containing canine fibroblast A-72 cells (test wells). Wells were also established to represent a cell control (cells that were not exposed to either CPV or TF) and virus control (cells exposed to CPV following incubation with culture medium). After incubation of the cells for 12 days, the supernatants from the cell control, virus control and TF treated wells were assayed for *in vitro* virucidal activity using a virus-related hemagglutination assay. To determine whether TF was directly cytotoxic to the canine fibroblasts, cells were plated and exposed to dilutions of TF (1 pg/ml to 10 mg/ml). After incubating for 12 days, the wells were assessed for cellular proliferation using a Microculture Tetrazolium Assay [Delhaes et al., 1999].

Erythrocyte hemolysis

To test the TF for erythrocyte hemolysis, canine erythrocytes were collected and separated via centrifugation for four minutes at 2000 RCF (relative centrifugal force), the top two layers decanted, and the remaining erythrocytes washed three times with dPBS. dPBS was then added to the cells to make a 10% (v/v) suspension. TF was prepared in dPBS and added to concentrations ranging from 0.001 to 1.0 mg/mL and incubated for 30 minutes. A positive control consisting of erythrocytes completely hemolyzed by the addition of 0.2% (v/v) Triton X-100 was compared to erythrocytes in dPBS and measured spectrophotometrically at 415 nm [Chow et al., 2005].

Statistical analysis

All experiments were arranged in a completely randomized block design. A Kaplan-Meier survival analysis was conducted for data from each survival study utilizing the LIFETEST procedure in SAS for Windows version 9.2 (SAS Institute, Cary, NC). P-values were calculated using Log-Rank or Wilcoxon methods and p-values <0.05 were considered significant.

RESULTS

Evaluating the Immunomodulatory Activity of TF in a Murine Model

The efficacy of the serum derived TF formulation against bacterial challenge was assessed in mice administered 5,000 CFU of *S. enterica* ser. Typhimurium genetically altered to bioluminesce via insertion of a lux gene construct. After 48 hours the mice were examined and the control (placebo) group was found to present with a visibly higher titer of *Salmonella*. This was confirmed by noting a 2.5 log increase in bioluminescence emanating from harvested spleens and a 13,600 fold increase of CFU/g of spleen when compared to TF treated mice (Fig. 1). The bioluminescence imaged in the TF group most likely resulted from an exogenous contamination of the field when delivering the inoculum. Histological examination of spleens harvested from control and treated mice further confirm this

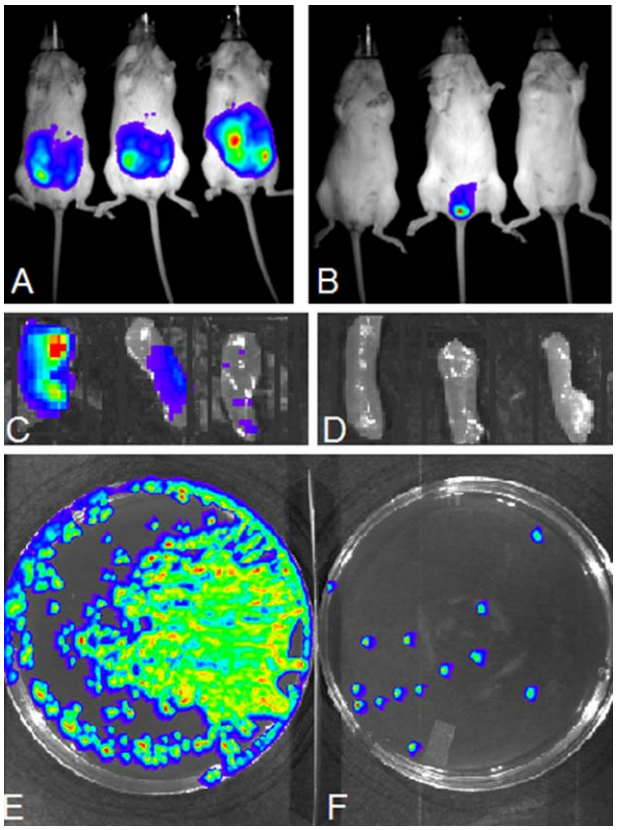

Fig. 1. Mice representing the Negative Control (A) and TF Treated (B) groups along with their harvested spleens (C, D) and splenic homogenates on Brilliant Green Agar + Ampicillin Sodium Salt, (2 μg/ml) (E, F) were imaged for bioluminescence emanating from *S. typhimurium* modified with the pAK1-lux plasmid construct 2 days after challenge. Mice were chemically restrained with isoflurane (1.5–3.0%) via nose cone and maintained on a 37°C heated platform to ensure stability while photonic emissions were recorded for 5 minutes with an IVIS 100 biophotonic imaging system (Xenogen Corporation). TF treated mice were provided 5 mg TF while control mice received a physiologic saline placebo, each as a 0.20 ml sc injection 24 hours before challenge with approximately 5×10^3 CFU/mouse. The agar plates represent homogenates that were prepared equivalently in that the dilutions as assessed on a per mg/ml basis were identical for both the control and TF treated samples in order to provide a visual comparator of bacterial presence. [Color figure can be viewed at wileyonlinelibrary.com]

observation as pockets of *Salmonella* were distributed throughout the spleens of mice not treated with TF while there was no evidence of splenic infection 48 hours post challenge in TF treated mice (Fig. 2). By day four control mice had over 1 million CFU/g of spleen and visible pockets of colonization. Over the course of the 1 week observation period, 93% of control mice succumbed to infection whereas 27% of the TF-treated mice succumbed (Fig. 3).

The serum of naïve and TF treated mice were analyzed for IL-6 and INF-γ. TF-treated mice demonstrated a rapid temporal increase in serum IL-6 and INF-γ concentrations. IL-6 increased from a baseline value of 5.5 pg/ml to 141 pg/ml in 3 hours (26-fold increase) and INF-γ increased from 7.8 to 597 pg/mL at 24 hours (77-fold increase) ($p < 0.05$) before returning to baseline. Interferon inducible protein 1 (Irgm1) was up-regulated in mice nearly 4-fold within 3 hours of TF administration.

Evaluating the Ability of TF to Improve CPV Survival

The severity of parvoviral enteritis was assessed for each animal upon admission to the study. All dogs demonstrated a reluctance to eat prior to admission and upon presentation. There was no difference between the control and treated groups based upon admittance assessment scoring (1.88 versus 1.84, respectively). The group outcomes (animal survival) are presented in Figure 4. Over twice as many dogs given TF survived (68% of the TF treated group survived versus 32% of the placebo group). This difference was significant ($p \leq 0.01$) when group comparison statistics were performed. Three dogs in the control population and five dogs in the TF treated group were admitted with secondary complications or a concurrent hookworm infection: Control Group – Giardia ($n = 1$), Coccidia ($n = 1$), and hookworms ($n = 1$); TF-Treated Group – Bordetella ($n = 1$), Coccidia/Giardia ($n = 1$), Coccidia/pneumonia ($n = 1$), pneumonia only ($n = 1$), and Giardia/Bordetella ($n = 1$). Of the animals in the control group only a puppy with hookworms survived while in the TF-treated population the two animals diagnosed with concurrent pneumonia succumbed. A significant difference comparing the survival of TF treated versus control animals was maintained if the animals that entered the study with secondary complications or if animals which succumbed within the first 24 hours of observation were removed from the study population (Fig. 4).

Cytotoxicity to TF was tested *in situ* by incubating canine fibroblast A-72 cells with TF up to 10 mg/ml. These cells showed no adverse effects as assessed by cell viability and proliferation after exposure to TF up to 10 mg/mL. TF did not function as a virucide to CPV as its infectivity and proliferation were unaffected by 4 hours exposures to TF (up to 1 mg/ml) and erythrocyte hemolysis was not observed at TF concentrations up to 1 mg/ml.

DISCUSSION

The prophylactic administration of TF in mice challenged with a potentially lethal dose of *Salmonella* improved the group survival (7% of the control mice survived while 73% of the TF treated mice survived). Survival corresponded with noted reductions

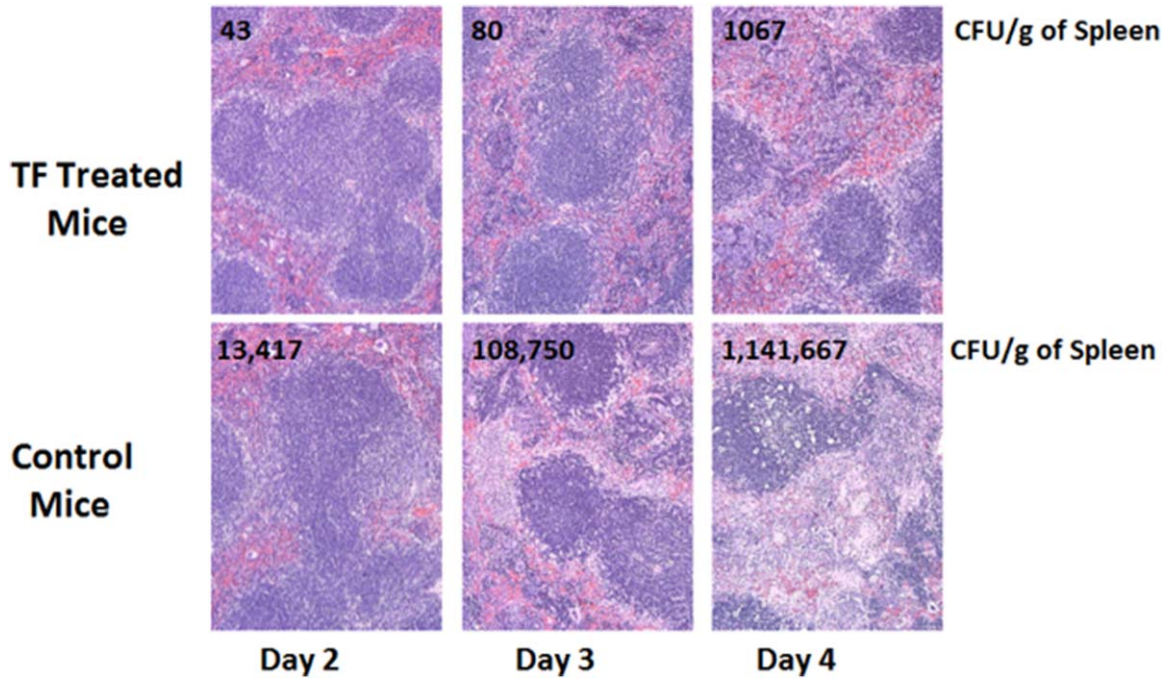

Fig. 2. Comparative Histological Slides prepared from the spleens of control and TF-treated mice inoculated with 5,000 cfu of salmonella on day 0. The white modeling apparent in day 3 and 4 control mice demonstrated bacterial colonization within the spleen—a clinical manifestation of systemic infection. Colony forming units (CFU) per gram of harvested splenic tissue is presented in their respective panels as the average of 3 replicates. [Color figure can be viewed at wileyonlinelibrary.com]

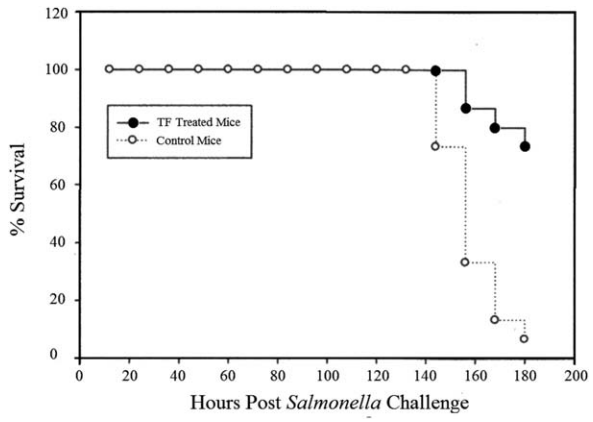

Fig. 3. Negative control mice ···○··· were challenged ip with 0.2 ml (~5 × 10^3 CFU) *S. typhimurium* on day 1, while TF treated mice–•–received both 0.2 mL (~5 × 10^3 CFU) *S. typhimurium* ip challenge on day 1 and a 0.2 mL TF (5 mg) subcutaneously on day 0. Each data point represents the cumulative percent survival ($n = 30$). Group differences were significant ($p = 0.01$).

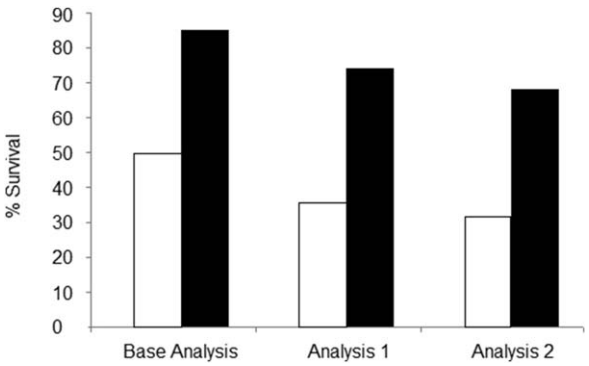

Fig. 4. Survival analysis of canines treated with TF following diagnosis with parvoviral enteritis. Dogs received supportive care and either 0.5 mL PBS subcutaneously (control group; white bars; $n = 25$) or 5 mg TF in 0.5 mL PBS subcutaneously (TF Treatment group; black bars; $n = 25$). Base analysis represents survivability of dogs without exclusion of individuals presenting with severe diarrhea or early mortality ($p = 0.010$). Analysis 1 represents total survivability after removal of individuals from the study due to mortality within 24 hours post admission ($p = 0.006$). Analysis 2 represents total survivability after removal of individuals from the study that presented with severe diarrhea upon admission to the study ($p = 0.015$).

in CFUs present in splenic tissue, a sentinel organ for systemic infection. TF increased expression of IL-6, a cytokine involved in host elimination of gram negative pathogens such as *Salmonella* [Weinstein et al., 1997]. Leukocyte-derived TF can act as an adjuvant prompting a heightened immune response to porcine parvovirus [Wang et al., 2012]. It was of interest to determine whether TF would also provide benefit against viral infection, CPV, when utilized in a veterinarian clinic. Here, animals were confirmed to have CPV based on fecal ELISA analysis and presentation symptoms. Animals were not uniformly

inoculated with CPV so the degree of CPV progression cannot be considered uniform. However, the random assignment of animals (even littermates) to treated and control groups assists in establishing how utilizing an immunotherapeutic approach could provide benefit to a clinical outcome.

TF was thus further tested in this study as a therapeutic agent for parvoviral enteritis. We examined the ability of TF to directly kill CPV (i.e., *in vitro* virucidal activity). In order to accurately assess this it was necessary to first establish whether the viability of the canine fibroblast A-72 host cell line was impaired by the presence of TF. Cell proliferation was not affected adversely when exposed to TF at concentrations up to 10 mg/ml, nor did exposure to TF up to 1 mg/ml induce hemolysis in isolates of canine erythrocytes. These observations provide some indication as to the overall safety of this preparation. TF was not virucidal to CPV as infectivity and proliferation were not affected by exposure to TF (up to 1 mg/ml). Group survival to parvoviral enteritis however increased by 36% when TF (a single 5 mg dose) was part of the therapeutic regime.

It is clear from the data reported that TF promotes or assists in resistance to CPV infection without acting as a virucidal agent. The mechanism by which it accomplishes this task remains to be elucidated but its overall characteristics categorize it as a biological response modifier. It is equally important to acknowledge that in part these actions are mediated via changes in cytokine profiles. Recombinant feline interferon-Ω has also been observed to significantly reduce mortality due to canine parvoviral infection/enteritis [Minagawa et al., 1999; Martin et al., 2002]. It is likely that it promotes its effect through the antiviral and immunomodulatory activities of interferon. An immune response capable of reducing levels of a systemically infected bacterium, such as the one initiated by TF would be expected to alter protein expression. The ability of TF to regulate the expression of several key biomarkers was monitored in murine serum. Interferon inducible protein 1 (Irgm1) was up-regulated in mice nearly fourfold within 3 hours of TF administration while serum amyloid A (SAA) which is normally present in the serum at low concentrations (1–5 μg/mL) was up-regulated by approximately 3-orders of magnitude within 48 hours post administration of TF [Parker et al., 2011]. The up-regulation of both Irgm1 and SAA help advance our understanding of the immunotherapeutic properties of TF within our model system. The opsinozation and increased phagocytosis induced by SAA in conjunction with possible increased elimination of phagocytized bacteria from up-regulation of the IFN-gamma effector molecule Irgm1 provides a detailed and working hypothesis for a mode of action associated with TF's immune modulating properties for the sequestering and elimination of *S. enterica* ser. Typhimurium. Herein we also demonstrate the direct temporal influence of TF on the expression of INF-γ, a lymphokine/cytokine acknowledged to help eliminate viral presence [Lanford et al., 2003]. The expression of INF-γ was increased over 70-fold when inspected at 24 hours post TF administration.

Mortality associated with CPV infections rarely results from the virus itself in individuals infected prior to 8 weeks of age [McCaw and Hoskins, 2006]. Mortality is usually proceeded by hemorrhagic enteritis, myocarditis, secondary infections, and gram negative sepsis [Wessels and Gaffin, 1986; Evermann et al., 2005; McCaw and Hoskins, 2006], followed by disseminated intravascular coagulation and death. Pathogenesis resulting in death in dogs over 8 weeks of age may take one of two routes; CPV infections of lymphoid tissue and marrow or infection within epithelial cells particularly within the crypts of the small intestines [Favrot et al., 2000]. Both routes typically result in secondary infection and gram-negative sepsis. TF intercedes in these processes by either interfering with viral pathogenesis, moderating the secondary effects resulting from immunodeficiency (i.e., secondary infection), or via modulation of both these activities. This study demonstrates that while TF is not a contact virucide (parvoviral infectivity and proliferation were not diminished after an isolated exposure to TF), its administration to CPV infected puppies reduced group mortality significantly. While TF has been shown to reduce mortality associated with microbial pathogens this is the first study which demonstrates that TF abates viral pathogenicity, likely facilitated through the antiviral characteristics of INF-γ.

Discovering agents that potentiate the immune response is a driving force in modern bio-rational drug research [Bacha et al., 2004]. Cytokinal factors such as the interferons currently in use may provide dramatic therapeutic results but also require therapeutic dosages at concentrations that often produce toxic side effects, thus making them intolerable to many patients which thereby limits their wide-spread general use [Kruth, 1998; Nasraway, 2003]. TFs may prove to be an attractive alternative. TFs and immunostimulants are widely marketed but not well regulated. The serum derived TF herein is a research preparation and not commercially available but is likely to be found in a USDA approved immunostimulant identified as PulmoClear™ (Colorado Serum Company, Denver, CO). Previous research demonstrated that it provided similar benefits to *Salmonella*-infected mice

[Willeford et al., 2001]. PulmoClear is derived from caprine serum but possesses high molecular weight proteins (e.g., IgG and albumin) that could initiate a site reaction or produce delayed hypersensitivity reactions upon recurrent use.

CONFLICTS OF INTEREST

The authors declare no conflict of interest or financial participation.

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