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Evaluation of protection in a mouse model after vaccination with *Mycobacterium avium* subsp. *paratuberculois* protein cocktails

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ABSTRACT

Whole-cell vaccines successfully reduce signs of clinical disease and fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), however, these vaccines have some limitations. The present study was conducted to identify MAP proteins that might be candidates for the development of an improved vaccine. MAP proteins were screened for immunogenicity in naturally infected cattle and selected based upon reactivity in the interferon-γ (IFN-γ) and Western blot assays. Proteins (MAP1087, MAP1204, MAP1272c, and MAP2077c) were arrayed into 4 overlapping cocktails containing 3 proteins each. The efficacy of the proteins within these cocktails as vaccine candidates was evaluated by subcutaneous immunization of mice, followed by challenge with live, virulent MAP. All MAP protein cocktails significantly reduced the recovery of live MAP from the ileum, while cocktails 1 and 3 reduced colonization in the liver. No significant differences were seen in the mesenteric lymph node or spleen, however, cocktail 1 reduced viable MAP in the mesenteric lymph node compared to other treatments. Stimulation of splenocytes upregulated antigen-specific IFN-γ and IL-23 secretion in all treatment groups, regardless of vaccination. Interestingly, IL-4 was moderately downregulated for vaccinates compared to control infected mice. An increase in total CD25 expression was noted for 3 of the 4 vaccinate groups upon stimulation of splenocytes with a whole cell sonicate of MAP, with this effect becoming more significant within CD4CD25+ and CD8CD25+ subpopulations. The present study demonstrated that MAP proteins are useful as vaccine candidates to reduce MAP tissue burden.

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1. Introduction

Paratuberculosis vaccine studies have demonstrated the induction of both cellular and humoral immune responses, however, it is widely accepted that vaccination will not prevent infection. Some benefits of vaccination include reduced fecal shedding of MAP and reduced clinical signs in infected animals, with evidence suggesting a reduction in the incidence of disease within herds or severity of disease for individual animals [1–3]. The heat-killed whole cell vaccine that is approved for use in the US (Mycopar, Fort Dodge Animal Health) is not ideal because of potential adverse reactions, including severe inflammation and granuloma formation at the injection site. In addition, vaccination with whole cell vaccines has been shown to interfere with bovine tuberculosis skin testing and serologic detection of MAP infected animals [4–6]. Developing subunit or DNA vaccines would significantly reduce or eliminate some of the troubling aspects of the whole cell vaccine without sacrificing beneficial properties.

Several MAP proteins or protein complexes have demonstrated success for use as subunit vaccines, including a 70 kDa heat shock protein, a novel 74F polyprotein, and a mixture of Ag85/SOD proteins. Immunization with these protein or protein complexes has provided protection against MAP challenge in mice, cattle and goat models, resulting in reduced colonization of tissues and decreased shedding in the feces [7–9]. Each of these subunit vaccines has demonstrated that they are able to induce both cell-mediated and humoral immune responses in the respective hosts, suggesting strong protective measures. Further, it was recently demonstrated that the Hsp70 subunit vaccine does not cross-react with the comparative cervical skin test, a diagnostic tool commonly used for bovine tuberculosis in the field [10]. Positive responses to AvPPD were noted in all vaccinated animals, however, responses to BoPPD were demonstrated only for cattle vaccinated with whole cell vaccine (Gudair) and not for those vaccinated with Hsp70 [10], demonstrating that a subunit vaccine can be more discriminative for identification of animals infected or vaccinated against MAP versus those animals infected with *Mycobacterium bovis*.

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Table 1
Mycobacterium avium subsp. *paratuberculosis* proteins.

NCBI gene ID[a]	Locus tag	Size (kDa)	Predicted function	*M. avium* homolog[b]	Vaccine pools
2719512	MAP1087	15.4	ABC transporter permease	MAV_3420	1, 2, 3
2720812	MAP1204	25.4	Putative invasion protein; NipC/P60 family; cell wall-associated hydrolase	MAV_3300	1, 2, 4
2720835	MAP1272c	33.4	Putative invasion protein; NipC/P60 family; cell wall-associated hydrolase	MAV_3208	1, 3, 4
2719950	MAP2077c	11.1	STAS[c] domain containing protein	MAV_2109	2, 3, 4

[a] A unique identification number for sequences in the NCBI database.
[b] The locus tag for the corresponding protein in *M. avium* subspecies *hominissuis* strain 104.
[c] STAS is an acronym for sulfate transporter antagonist of anti-sigma factor.

Disadvantages of protein vaccines are that purified antigens often do not stimulate robust or durable immune responses, requiring adjuvants to enhance effectiveness. Adjuvants used in veterinary vaccines are often based upon fragments of mycobacteria and other bacteria or chemicals that elicit strong inflammatory responses to stimulate immunity [11]. The use of adjuvants with protein-based vaccines can reduce the amount of antigen needed, as well the number of immunizations required to achieve protective immunity. In the present study, cocktails of MAP proteins previously shown to elicit strong immune responses in cattle were evaluated for host immunity and potential use as vaccine candidates for protection against MAP infection in a mouse model.

2. Materials and methods

2.1. Antigen screening

Four MAP proteins were evaluated for reactivity with control noninfected, subclinically and clinically infected cattle in a whole blood interferon-γ (IFN-γ) assay. Infection status of cattle was confirmed by bacteriologic culture for the fecal shedding of MAP and serologic assays by standard methods [12,13]. Whole blood was incubated with medium only (nonstimulated; NS), concanavalinA (ConA, Sigma), pokeweed mitogen (PWM; 10 μg/ml; Sigma), johnin purified protein derivative (JPPD; 10 μg/ml; National Veterinary Services Laboratory, Ames, IA), a whole-cell sonicate of MAP (MPS; 10 μg/ml; NADC), and one of the following MAP proteins, MAP1087, MAP1204, MAP1272c, MAP2077c, (Table 1; 10 μg/ml). After incubation at 39 °C for 18 h, plasma was assayed using a Bovigam IFN-γ assay (Prionics, La Vista, NE). The selection of the 4 MAP proteins, MAP1087, MAP1204, MAP1272c, and MAP2077c, used in the present study was based upon their immunogenic potential as defined by robust antigen-specific IFN-γ and antibody responses in infected cattle and negligible responses in noninfected control cows. These 4 proteins were arrayed in 4 cocktails containing 3 of the 4 proteins as follows: cocktail 1: MAP1087, MAP1204, MAP1272c; cocktail 2: MAP1087, MAP1204, MAP2077c; cocktail 3: MAP 1087, 1272c, 2077c; and cocktail 4: MAP1204, MAP1272c, 2077c.

2.2. Protein expression and purification

The four annotated coding sequences of the proteins were selected from a battery of recombinant proteins amplified from strain K-10 genomic DNA. The methods for expression and purification of MAP recombinant proteins are described in detail [14]. 74F polyprotein, consisting of a combination of MAP1519 and MAP3527 peptides, was constructed as previously described [8]. The 74F polyprotein had previously demonstrated efficacy in the mouse model as a vaccine for paratuberculosis [8].

2.3. Bacterial strain

MAP used as the challenge strain was isolated from the ileum of clinical cow 167 (NADC) and was grown in Middlebrook 7H9 liquid medium (pH 5.9) supplemented with 0.5% Tween 80 (Sigma), 2 mg/ml mycobactin J (Allied Monitor Inc., Fayette, MO) and 10% oleic acid–albumin–dextrose complex (BD Biosciences, Franklin Lakes, NJ). The final concentration of the bacteria was adjusted to 10^9 cfu/ml and confirmed by serial dilution onto agar slants of Herrold's egg yolk medium (HEYM; BD) containing 2 mg/l of mycobactin J (Allied Monitor) with a final read-out after 12 weeks of incubation. The presence of each of the 4 MAP proteins in the cocktails was confirmed in this strain by PCR and Western blot (data not shown). Clinical cow strain 167 was used as the challenge strain as it was expanded from a primary isolate from ileal tissue of a highly infected cow and had not been subjected to repeated passage in laboratory medium.

2.4. Mice

Six-week old, male Balb/c mice used in the study (Jackson Labs, Bar Harbor, Maine) were housed in biosecurity level-2 containment in disposable plastic cages with free access to water and standard mouse chow. All procedures were approved by the NADC Animal Care and Use Committee.

2.5. MAP vaccines

Mice were randomly assigned to 7 treatment groups containing 10 mice each as follows: control uninfected (no vaccine, no MAP), control infected (no vaccine, MAP infection), 74F protein only (74F vaccine, MAP infection), cocktail 1 (cocktail 1, MAP infection), cocktail 2 (cocktail 2, MAP infection), cocktail 3 (cocktail 3, MAP infection) and cocktail 4 (cocktail 4, MAP infection). The 74F group received 50 μg total protein as described in a previous study [8] and mice in cocktail 1–4 groups were given 100 μg total protein in 100 μl volume per mouse subcutaneously (SQ) in the dorsal region. The control uninfected and control infected groups received 100 μl PBS as sham injections. Mice within each treatment group were boosted with the identical vaccine 3 weeks after the initial immunization. Two weeks after boosting, mice were inoculated intraperitoneally with live, virulent MAP strain 167 (10^8 in 100 μl). Three months after infection, mice were anesthetized by inhalation of isoflurane and decapitated with a guillotine. The liver, spleen, ileum and mesenteric lymph node were removed from each mouse, weighed, and processed for tissue culture as previously described [15]. Splenocytes were isolated from a portion of the spleen and cultured at 2.0×10^6 cells/ml with medium only (NS); ConA, 10 μg/ml; PWM, 10 μg/ml; and MPS, 10 μg/ml. Quantitative cytokine analyses was performed on 24 h supernatants for interleukins (IL)-2, IL-4, IL-10, IL-12, IL-23 and IFN-γ using commercial ELISA kits, according to accompanying protocol (R & D Systems, Minneapolis, MN). After 6 days of incubation, splenocytes were harvested for flow cytometric analyses of CD3, CD4, γδ T cells (BD Biosciences), CD8, B cells, monocytes, CD44, CD62L, and CD25 expression (BioLegend, San Diego, CA). Data analyses were performed using FlowJo software (TreeStar, Inc., San Carlos, CA).

Antigen-specific IgG_1 and IgG_{2a} responses were measured in mouse sera by ELISA. Briefly, plates (Nunc MaxiSorp module, Nunc,

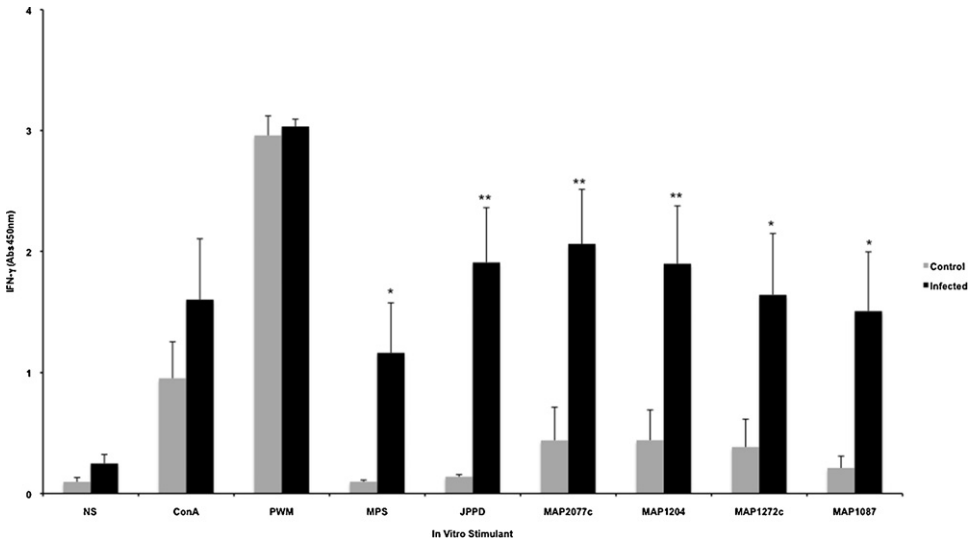

Fig. 1. Secretion of interferon-γ (Abs_{450nm}; IFN-γ) by control noninfected cows and cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* upon incubation of whole blood with medium alone (NS); concanavalinA (ConA); pokeweed mitogen (PWM); a whole-cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS); johnin purified protein derivative (JPPD); and MAP proteins (2077c, 1204, 1272c, and 1087). Data are expressed as means ± SEM. Significant differences between control and infection cows within in vitro treatment group are represented by asterisks ($^{**}P < 0.01$; $^{*}P < 0.05$).

Roskilde, Denmark) were coated with 200 ng/well of a whole cell sonicate preparation of MAP (clinical strain 167, NADC) and incubated at 4 °C overnight. After blocking (1% BSA in TBST), diluted sera were added to the wells and incubated at 25 °C for 1 h, followed by incubation with either horseradish peroxidase-conjugated goat anti-mouse IgG_1 or IgG_{2a} (Kamiya Biochemical Co., Seattle, WA) for 20 min, and 3,3′,5,5′-tetramethylbenzidine substrate solution (TMB; Kamiya Biomedical Co.) for 10 min. Absorbance at 450 nm was measured in a Victor X_3 Microplate reader (Perkin-Elmer, Shelton, CT).

2.6. Statistical analysis

Data were analyzed using PROC MIXED procedure of the Statistical Analysis System (SAS Inst., Inc., Cary, NC). The model included the fixed effects of treatment (vaccination), stimulation (in vitro treatment), and treatment × stimulation interaction. When significant effects ($P < 0.05$) due to treatment, stimulation, or treatment × stimulation interactions were detected, means separation was conducted by the Student's *t*-test option in SAS. Designation of statistical significance within figures is described in each figure legend. Broad measures of statistical significance due to treatment or treatment × stimulation interactions may only be denoted within the text of the manuscript.

3. Results

3.1. Immunogenicity of MAP protein candidates

The MAP proteins (MAP1087, MAP1204, MAP1272c, and MAP2077c) used in the present study to formulate vaccine cocktails demonstrated strong immunogenic potential, producing antigen-specific IFN-γ responses similar to or greater than the whole-cell sonicate of MAP (MPS) in Johne's subclinical and clinical cows (Fig. 1).

3.2. Cytokine results

Results for Th1 and Th2-mediated cytokine secretion from splenocytes stimulated with either medium alone (NS) or with MPS are presented in Fig. 2. Stimulation of cells with MPS resulted in an upregulation ($P < 0.05$) of IFN-γ compared to NS cultures in all infected groups. Vaccination with MAP protein cocktails reduced IFN-γ responses to MPS overall when compared to the control infected and 74F treatment groups ($P < 0.05$). Secretion of IL-12 followed a similar trend with greater responses noted for the control infected and 74F groups compared to the protein cocktail vaccinates (Fig. 2B). MPS-stimulated splenocytes had greater ($P < 0.05$) IL-4 responses compared to NS cultures only in control infected mice (Fig. 2C). Interestingly, immunization with MAP protein cocktails resulted in increased ($P < 0.05$) IL-4 secretion in NS cultures for mice immunized with cocktails 1 and 2 compared to control infected mice. There were no significant effects due to vaccination on the secretion of IL-10, regardless of in vitro stimulation, however ($P < 0.05$) differences between NS and MPS-stimulated cultures were observed for control infected mice (Fig. 2D). Secretion of IL-2 and IL-23 was not influenced by vaccination but an upregulation of IL-23 was observed by stimulation of splenocytes with MPS in all infected mice (data not shown).

3.3. T cell populations

Vaccination with protein cocktails 1–3 and 74F resulted in higher ($P < 0.05$) CD4T cells compared to control infected mice, regardless of in vitro treatment (data not shown). In addition, immunization of mice with MAP protein cocktails (1–3) or 74F resulted in dramatic ($P < 0.05$) upregulation of CD4CD25T cells in MPS-stimulated cultures compared to NS cultures (Fig. 3A and B). There were no major differences in CD8T cells due to vaccination of mice (data not shown), however, the number of CD8CD25T cells was significantly ($P < 0.05$) upregulated in MPS-stimulated splenocytes across treatment groups (Fig. 3C and D). There were no differences in the percentage of γδ T cells and γδCD25+ cells due to vaccination, yet there was a consistent trend toward reduced numbers of these cell types after MPS stimulation of splenocytes in all treatment groups (data not shown). There was a trend for increased total CD25T cells in mice vaccinated with MAP proteins or 74F although only mice in cocktail 4 had significantly ($P < 0.05$) higher CD25T cells after MPS stimulation of splenocytes than control infected mice (8.30 ± 1.76 versus 5.46 ± 0.70, respectively; data not shown). Total percentages of CD44, CD62L, and monocytes in

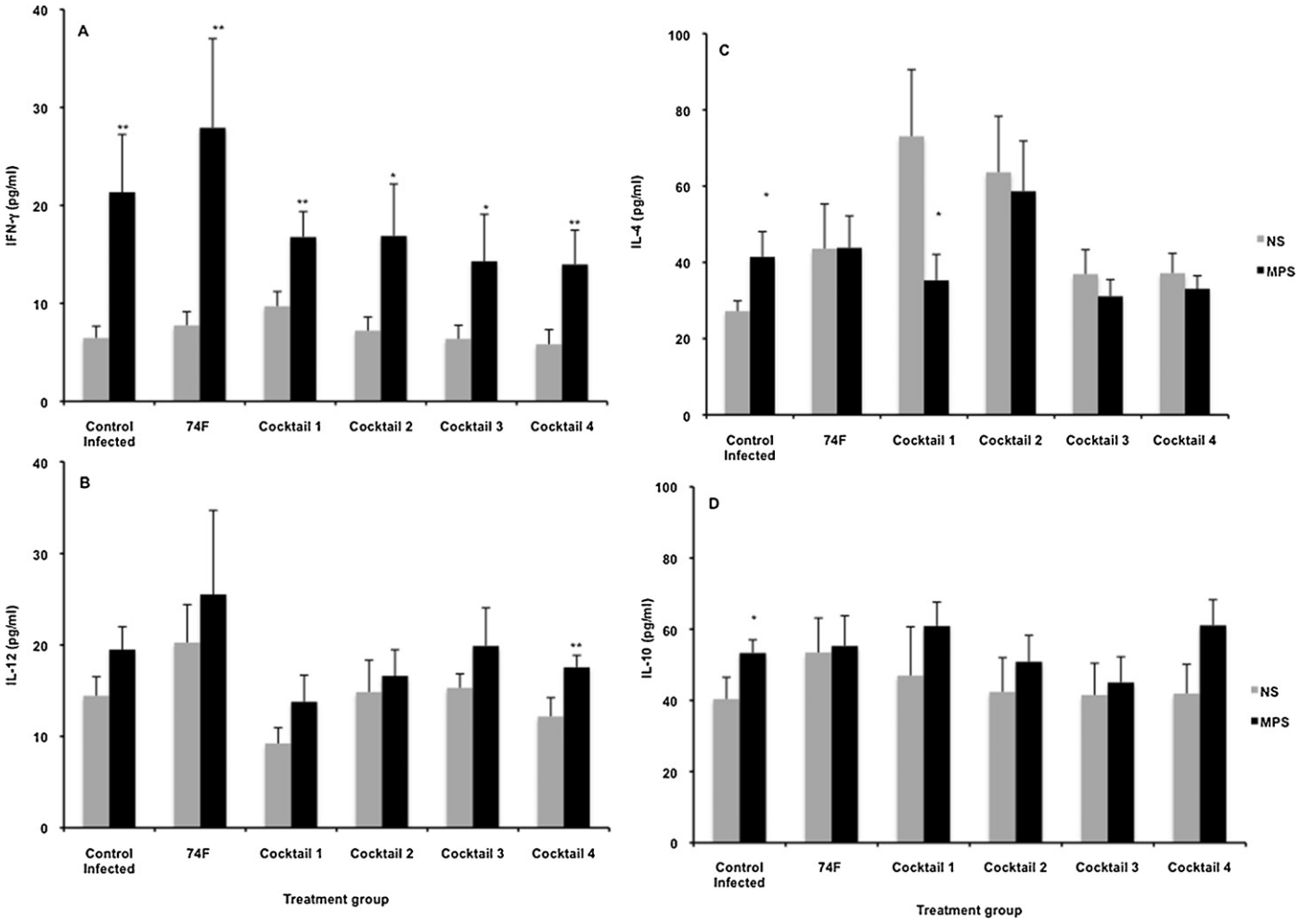

Fig. 2. Secretion of Th1-mediated cytokines, interferon-γ (A, IFN-γ; pg/ml) and interleukin-12 (B, IL-12; pg/ml), and Th2-mediated cytokines, interleukin-4 (C, IL-4; pg/ml), and interleukin-10 (D, IL-10; pg/ml) by splenocytes stimulated with medium alone (NS) or with a whole cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS). Splenocytes were isolated after 3 months of infection from control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1–4. Data are expressed as means ± SEM. Significant differences between NS and MPS within treatment group are represented by asterisks ($^{**}P < 0.01$; $^{*}P < 0.05$).

splenocyte cultures were unaffected by immunization of mice or in vitro treatment in the present study (data not shown).

3.4. B cells and MAP-specific serum IgG_1 and IgG_{2a} results

Interestingly, the percentages of B cells within MPS-stimulated splenocytes were lower ($P < 0.05$) in mice immunized with cocktails 1, 2, and 4, compared to control infected mice (data not shown). Infection with MAP with or without immunization resulted in significant ($P < 0.05$) increases in serum IgG_1 and IgG_{2a} compared to control noninfected mice (Fig. 4). Mice immunized with either 74F or the MAP protein cocktails demonstrated increased ($P < 0.05$) MAP-specific serum IgG_{2a} following challenge compared to control infected mice. In contrast, differences due to vaccination were not noted for MAP-specific serum IgG_1 antibodies with similar levels noted for all infected mice regardless of vaccination when compared to control infected mice.

3.5. Tissue culture

Immunization with cocktails 1–4 did not significantly impact tissue colonization in the spleen after challenge with live MAP, yet there was a trend toward reduced MAP colonization noted in the cocktail 1 group compared to the control infected group (Fig. 5A). Similar reductions in MAP colonization were also noted in the MLN of mice receiving cocktails 1 and 2 prior to challenge, although these differences did not achieve statistical significance (Fig. 5C). However, liver colonization was reduced ($P < 0.05$) in mice immunized with cocktails 1 and 3, as well as the 74F polyprotein that served as a positive control in the study (Fig. 5B). Further, all protein cocktails significantly ($P < 0.05$) reduced MAP colonization in the ileum compared to infected controls (Fig. 5D). Although immunization with the combination of proteins in cocktail 4 (MAP1204, MAP1272c, and MAP2077c) provided less protection against MAP colonization compared to the 3 other cocktails, it was still effective in reducing tissue burden in the ileum.

4. Discussion

The four MAP protein candidates were chosen based upon earlier work suggesting moderate to high antigenic responses in serologic assays. Functions of these proteins include a peptide transport system permease protein, MAP1087; putative invasion proteins, MAP1204 and MAP1272c; and a STAS domain containing protein, MAP 2077c. Two of the 4 selected proteins (MAP1087 and MAP1204) had previously demonstrated strong reactivity with sera from naturally infected cattle in the subclinical stage of infection [16]. In addition, MAP1087, MAP1204, and MAP2077c all reacted with sera from experimentally infected calves in the early stage of infection [16]. The selection of these proteins was not based upon

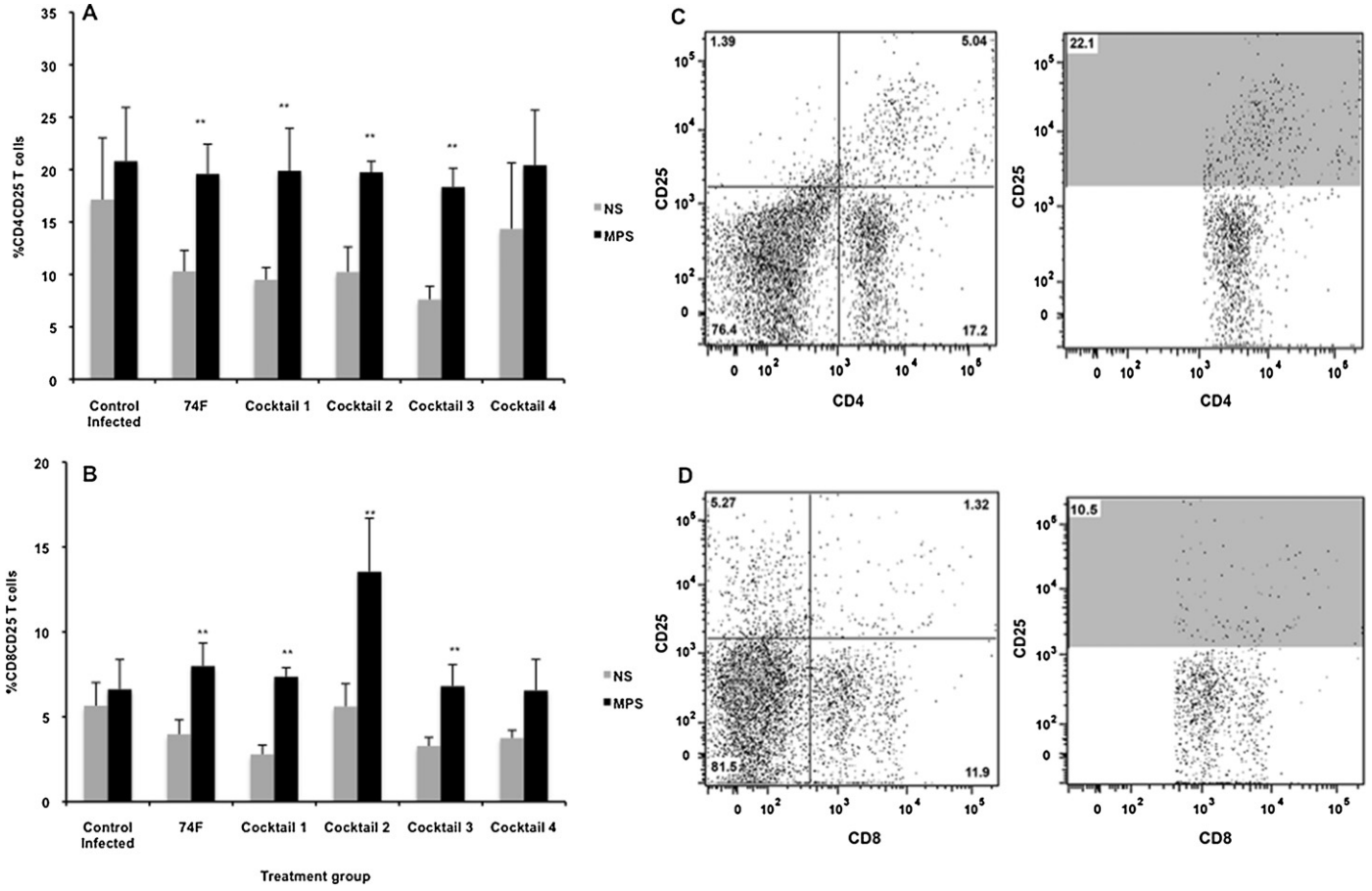

Fig. 3. Percentage of CD4CD25+ (A) and CD8CD25+ (B) T cells from splenocytes stimulated with medium alone (NS) or with a whole cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS). Representative dot plot profiles of CD4CD25+ (C) and CD8CD25+ (D) populations in MPS-stimulated splenocytes. Splenocytes were isolated after 3 months of infection from control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1–4. Data are expressed as means ± SEM. Significant differences between NS and MPS stimulants within a treatment group are represented by asterisks ($^{**}P < 0.01$; $^{*}P < 0.05$).

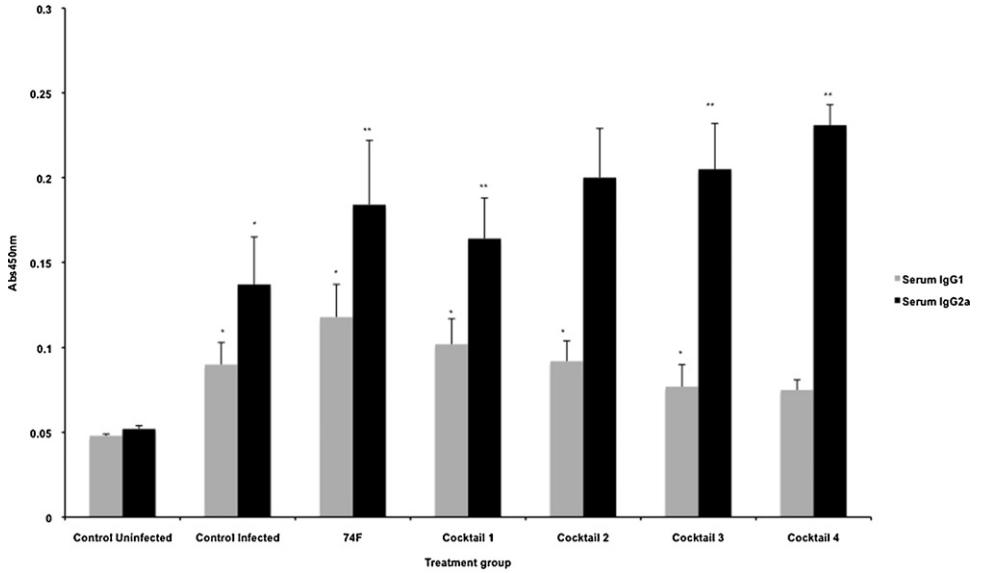

Fig. 4. Serum IgG_1 and IgG_{2a} antibody responses against *Mycobacterium avium* subsp. *paratuberculosis* after 3 months of infection from control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1–4. Data are expressed as means ± SEM. Significant differences between control uninfected, control infected and vaccinate groups are represented by asterisks ($^{**}P < 0.01$; $^{*}P < 0.05$).

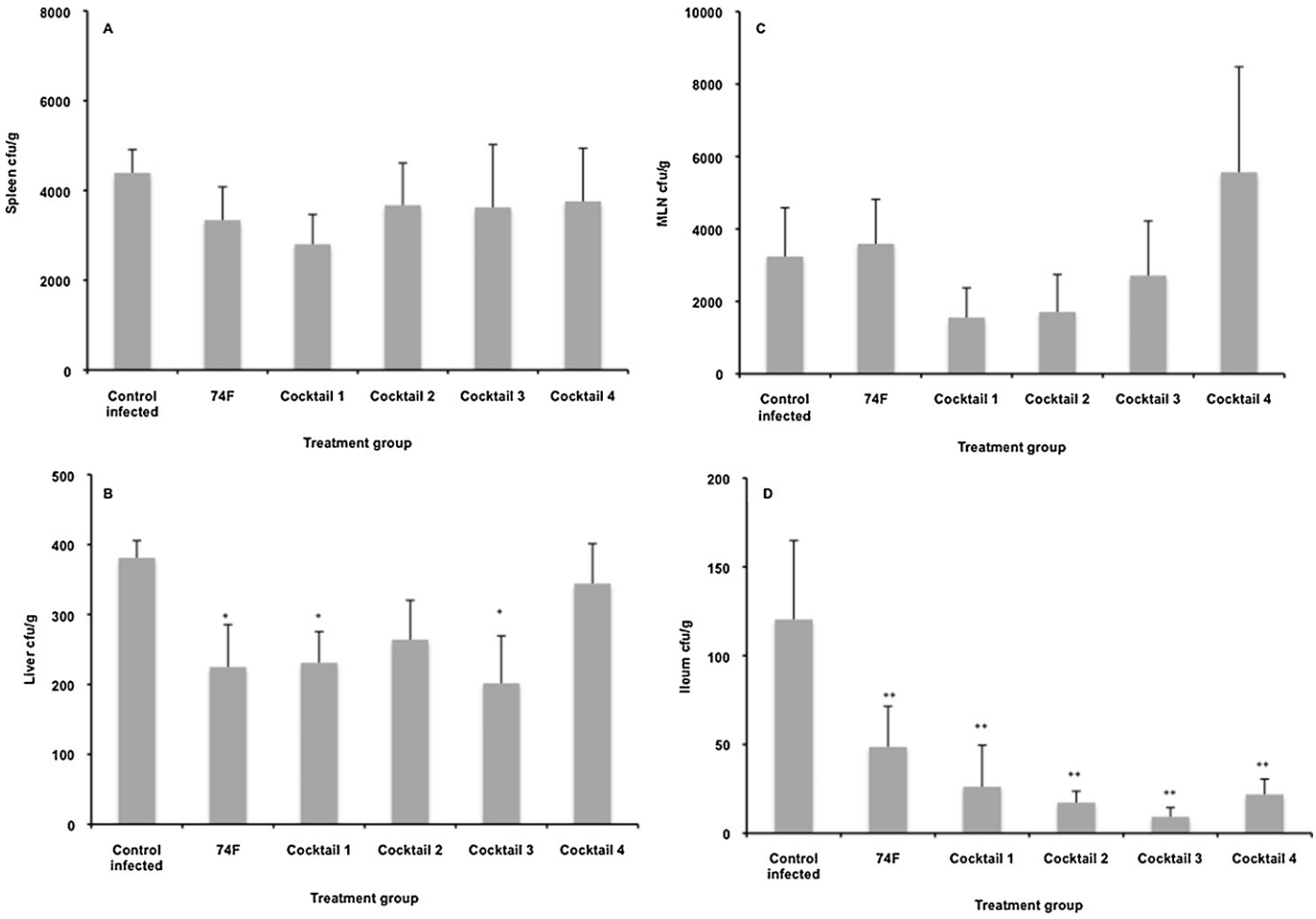

Fig. 5. Recovery of viable *Mycobacterium avium* subsp. *paratuberculosis* (cfu/g tissue) from (A) spleen, (B) liver, (C) mesenteric lymph node (MLN), and (D) ileum of control mice and mice vaccinated with 74F polyprotein or MAP protein cocktails 1–4 after 3 months of infection. Data are expressed as means ± SEM. Significant differences between control infected and vaccinate groups are represented by asterisks ($^{**}P < 0.01$; $^{*}P < 0.05$).

specificity to MAP and protein sequence analysis demonstrated significant alignment of all 4 proteins to both MAP and *Mycobacterium avium*. This is not surprising given the high level of genetic homology (>98%) between MAP and *M. avium*, with few unique genes identified for MAP and even fewer immunogens [14,17]. Although partial sequences of MAP1204 were also found in other mycobacterial species, including *M. bovis*, this would not be a major detractor from potential use as a vaccine candidate. Sero-diagnostic tests for the detection of *M. bovis* infection using antigens such as ESAT-6, CFP-10, and MPB83, have demonstrated a lack of cross-reactivity in calves vaccinated with the whole-cell vaccine for MAP [13]. In addition, IFN-γ responses to ESAT-6:CFP-10 antigens were observed to be highly specific for calves infected with *M. bovis*, with negligible reactivity noted for MAP- and *M. avium*-infected calves [18] or for calves vaccinated with a whole cell vaccine [13]. This would suggest that adequate tools are available to distinguish between MAP vaccinates and animals infected with *M. bovis*.

Although it is unknown how well immunogenic proteins translate into successful vaccines, it is understood that proteins can be highly antigenic, eliciting both humoral and cell-mediated immune responses. Many researchers utilize immune reactivity in the host as their primary method to screen protein candidates for subunit vaccines, with the hypothesis that an induced response is suggestive of protective immunity. Although proteomic and genomic screening approaches can be used to identify vaccine candidates, these tools are only useful if the candidates are recognized by the host immune system. Screening tools such as Western blotting have been effective for many bacterial pathogens, however, for intracellular pathogens such as MAP, antigen screening should include some measure of responsiveness in a cell-mediated assay since protection is aligned with Th1-mediated immunity in the host [19]. The proteins in the present study were selected upon observation of robust IFN-γ responses in naturally infected cattle, combined with the ability to discriminate between infected and noninfected cattle with some measure of specificity.

Numerous infection models have been developed for MAP, with emphasis on ruminant species such as cattle, sheep, goats, deer, and bison [20] since these are the target species for paratuberculosis. However, the protracted period of subclinical infection that occurs in naturally infected hosts is mimicked in experimentally infected animals, resulting in lengthy study periods [20,21]. Mouse models provide a reasonable approach to efficiently evaluate vaccine candidates due to a shorter infection periods compared to ruminants, greater reproducibility due to more precise genetics amongst treatment animals, and reduced costs for care and housing, allowing for greater numbers of animals per treatment group. Mouse models for MAP infection have been adequately characterized and IP infection of Balb/c mice results in effective colonization of the major target tissues [22].

Th1-mediated immune responses may be indicative of exposure to mycobacterial pathogens, including MAP, but also appear to be essential to keep infection from progressing from

subclinical to clinical disease. In a neonatal calf infection model, we were able to demonstrate the upregulation of immune markers including, robust antigen-specific IFN-γ responses as well as induction of antigen-specific CD25, CD26, and CD45RO expression less than 3 months after infection [23]. Although Th2 responses are not known to be protective in the host, both Th1- and Th2-mediated immunity has been induced after vaccination with a whole cell preparation of MAP [24–26]. Recent studies have also demonstrated that MAP vaccines comprised of single proteins or protein complexes will also evoke strong Th1 responses [7–9,26–28]. In the present study, immunization of mice with MAP protein cocktails prior to challenge with live MAP resulted in similar induction of antigen-specific IFN-γ when compared to control infected mice, although some attenuation of the IFN-γ response was noted. Differences in IFN-γ secretion between NS and MPS-stimulated cultures were lower for mice vaccinated with protein cocktails, particularly cocktail 1. This would suggest that immunization with this triad of proteins may have resulted in greater constitutive secretion of IFN-γ but lower antigen-specific IFN-γ secretion after challenge, an effect that may be advantageous to the host. Pro-inflammatory effects of IFN-γ can be both beneficial and detrimental to the host and a finite balance must be achieved in order to maintain effective immunity [28]. Interleukin-23 was upregulated by MAP infection, with increased secretion noted after stimulation with MPS antigen in vitro for all treatment groups. This is the first report describing effects of MAP infection on the secretion of IL-23. IL-23 is a newly recognized cytokine that is involved in the inflammatory response to mycobacterial infections, although little is known about its role in the immunopathology of MAP infections. IL-23 is required for the generation of effector memory T cells and is also needed for generation of IL-17-producing T cells, which play an important role in the inflammatory response [29].

Although Th2-mediated cytokine production was not markedly different due to vaccination, an interesting observation was the pattern of IL-4 secretion noted between NS and MPS-stimulated splenocytes in mice vaccinated with MAP protein cocktails, particularly for mice immunized with cocktail 1. Vaccination with MAP proteins 1087, 1204, and 1272c, in cocktail 1 resulted in higher constitutive secretion of IL-4 compared to the other treatment groups, something that was not apparent upon further exposure of splenocytes to MAP antigen in vitro. These results would suggest that this cocktail of MAP proteins modulated Th2 responses of the host after infection, an important consideration for selection of vaccine candidates. This is further substantiated by the increase in MAP-specific serum IgG_{2a} noted for mice immunized with either 74F or MAP protein cocktails. Secretion of IgG_{2a} antibodies is most closely associated with Th1-mediated immunity and cytokines such as IFN-γ, IL-4, and IL-10 can influence the isotype switch to IgG_{2a} [30].

Immune responses to vaccination are critical for the control of infection in the host. Immunization with the MAP protein cocktails effectively reduced MAP colonization of the liver and ileum. In particular, cocktail 1 also demonstrated efficacy for reduced colonization of the spleen and mesenteric lymph node, providing the most consistent effect on retardation of infection in the host. Reduced colonization of tissues is a beneficial characteristic for paratuberculosis vaccines and has been reported for commercial vaccines such as Mycopar and Gudair, as well as for more recently developed subunit vaccines [7,9,31,32]. Decreased tissue burdens result in reduced shedding of MAP in the feces thereby allaying spread of infection within a herd [1]. Similar reductions in tissue burden have been reported for mice immunized with the 74F polyprotein, with reduced recoveries of viable MAP from spleen, liver and mesenteric lymph nodes at 12 and 16 weeks post-challenge [8]. The highly positive results achieved with the 74F polyprotein prompted us to incorporate it into our study as a positive control. The Mycopar vaccine was an undesirable choice as a positive control vaccine in the current study as effects have not been previously evaluated in a mouse model. In addition, the Mycopar vaccine typically causes large granulomatous nodules at the injection site in ruminants, an effect that would not be handled well in a young mouse [31]. In ruminants, this local inflammatory effect is managed somewhat by injecting the vaccine in the fatty area of the brisket. Much of the benefit previously noted for the 74F immunogen was repeated in the present study, with concurrent reductions in tissue colonization and activation of T cells as previously described. Despite this, cocktail 1 seemed to invoke the most consistent responses in protection against tissue colonization. Further, this triad of MAP proteins, 1087, 1204, and 1272c, appeared to more tightly regulate the immune response post-immunization both before and after challenge with live MAP. Host responses after immunization with either cocktail 2 or 3 more closely aligned themselves to results observed for cocktail 1. The common protein between these 3 cocktails was MAP1087, whose known function is a peptide transport system permease protein. This type of transport protein has been considered as a potential vaccine for a wide variety of bacterial species [33]. This provides us with an interesting set of proteins to further evaluate as a potential MAP vaccine in a ruminant model.

In summary, the present study evaluated cocktails of MAP proteins as potential subunit vaccines for paratuberculosis. Cocktails of MAP proteins proved effective in protection against tissue colonization and invoked cell-mediated and humoral immunity in the host. Further evaluation of the components of cocktail 1, (MAP proteins 1087, 1204, and 1272c), needs to be undertaken to evaluate the potential of these proteins as subunit vaccines against Johne's disease.

Conflict of interest

The authors, J.R. Stabel and J.P. Bannantine, are employees of USDA-ARS, and USDA-ARS has filed a patent application for this work.

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