2014

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# Lymphoproliferative and Gamma Interferon Responses to Stress-Regulated *Mycobacterium avium* subsp. *paratuberculosis* Recombinant Proteins

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**Johne's disease in ruminants is a chronic infection of the intestines caused by *Mycobacterium avium* subsp. *paratuberculosis*. An important strategy to control disease is early detection, and a potentially efficient method for early detection is measurement of cell-mediated immune responses developed by the host in response to exposure or infection. One method is to measure lymphoproliferation and cytokine release from the host cells when exposed to the organism or parts of the organism. In this study, 10 recombinant *M. avium* subsp. *paratuberculosis* proteins known to be upregulated under *in vitro* stress conditions were evaluated by examining their ability to evoke memory as a result of exposure by vaccination or oral challenge with live *Mycobacterium avium* subsp. *paratuberculosis*. Out of 10 proteins, MAP2698c was found to induce higher cell-mediated immune responses in vaccinated and challenged sheep in comparison to healthy controls. The findings suggest that not all stress-regulated proteins have the diagnostic potential to detect cell-mediated immune responses in ovine paratuberculosis.**

Johne's disease (JD) in ruminants is a chronic infection of the intestines caused by *Mycobacterium avium* subsp. *paratuberculosis*. In sheep, economic losses arise due to reduced production of wool and lambs, mortalities, and culling. The disease is characterized by a long incubation period, and subclinical infection creates a potential source of infection for uninfected animals. Animals are infected with *M. avium* subsp. *paratuberculosis* early in life by the fecal-oral route from contaminated pasture, *in utero* infection, suckling infected dams (1, 2, 38), and aerosols (3). An important strategy required for the management and control of JD is early detection and culling of infected animals from the population. Subclinically infected animals may have intermittent patterns of *M. avium* subsp. *paratuberculosis* shedding; therefore, a confirmative test by fecal culture and PCR may be of low diagnostic value in the early stage of infection.

Following the ingestion of *M. avium* subsp. *paratuberculosis* via contaminated feed, mycobacteria invade the gut mucosa and are phagocytosed by antigen-presenting cells (APCs)—macrophages and dendritic cells. Infected cells migrate to regional lymphoid tissues and interact with lymphocytes to initiate adaptive immune responses. Following antigen presentation, the T cells are primed and then proliferate and differentiate into effector cells, producing important cytokines such as gamma interferon (IFN-γ) required for mounting cell-mediated immune responses to prevent progression of the disease into the clinical stage (4, 5). On subsequent exposure to the same antigen, primed immune cells are activated, proliferate, and produce IFN-γ as an effector to clear infection in a more vigorous manner than in the initial exposure.

A potentially efficient method for detection of early infection is by measuring cell-mediated immune responses developed by the host in response to exposure or infection. IFN-γ is a key cytokine produced by both αβ and by γδ T cells that upregulate major histocompatibility complex class II molecules (6) and has been used as an indicator of infection (7). This cytokine, secreted by activated and proliferating T cells postexposure, may be exploited for early diagnosis of JD.

Commonly used antigens to stimulate lymphocytes in whole blood to elicit *M. avium* subsp. *paratuberculosis*-specific cell-mediated immune responses have been *M. avium* subsp. *paratuberculosis* whole-cell lysates, purified protein derivatives (PPDs), or French-pressed antigens (8). The specificity of these crude whole-cell-derived antigens tends to be low (9–13). Therefore, investigation of *M. avium* subsp. *paratuberculosis* antigens with potential immunogenicity for diagnostic efficiency is required.

Several *M. avium* subsp. *paratuberculosis* proteins are differentially regulated in response to *in vitro* physiological stress conditions, similar to stress conditions encountered during intracellular survival in the host macrophage (14–16). *M. avium* subsp. *paratuberculosis* may express these stress proteins *in vivo* to evade host defense mechanisms, and the host may mount an early immune response to these antigens. Furthermore, proteins MAP2768c and MAP3273c were reported to play important roles in *M. avium* subsp. *paratuberculosis* latency and have the ability to induce IFN-γ production in *M. avium* subsp. *paratuberculosis*-infected cattle (17). Therefore, the cell-mediated immune response mounted by the host against other *M. avium* subsp. *paratuberculosis*-specific stress-regulated proteins may be important to explore for diagnostic potential.

The aim of this study was to evaluate the lymphocyte proliferation and IFN-γ induction ability of stress-regulated *M. avium* subsp. *paratuberculosis* recombinant proteins in sheep that were

**Received** 3 December 2013 **Returned for modification** 29 January 2014
**Accepted** 28 March 2014
**Published ahead of print** 2 April 2014
**Editor:** P. P. Wilkins
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doi:10.1128/CVI.00775-13

**TABLE 1** Recombinant antigens used in whole-blood stimulation for IFN-γ production and lymphoproliferation in PBMCs

| Recombinant tag | Antigen | Function | % homology in strain[a]: | | |
|---|---|---|---|---|---|
| | | | K-10[b] | 104[c] | ATCC 25291[d] |
| His tag | MAP2698c | Acyl-acyl carrier protein desaturase | 100 | 99 | 99 |
| | MAP3567 | Hypothetical protein or possible reductase | 100 | 99 | 99 |
| MBP fusion | MAP0187c | Superoxide dismutase | 100 | 99 | 99 |
| | MAP2487c | Carbonic anhydrase | 100 | 99 | 99 |
| | MAP3393c | IMP biosynthesis | 100 | 99 | 99 |
| | MAP3268 | Heat shock proteins 18_3 and 18_2 | 100 | 99 | 99 |
| | MAP1560 | Catalytic activity with thioesterases | 100 | 100 | 99 |
| | MAP1588c | Alkyl hydroperoxide reductase D | 100 | 99 | 100 |
| | MAP1589c | Alkyl hydroperoxide reductase C | 100 | 99 | 99 |
| | MAP1017c | Fatty acid metabolism | 100 | 99 | 99 |

[a] Sequence homology of all 10 proteins to *Mycobacterium tuberculosis* H37Rv was <90%. Protein sequence homology was as previously reported (19).
[b] *M. avium* subsp. *paratuberculosis* K-10.
[c] *M. avium* subsp. *hominissuis* 104.
[d] *M. avium* subsp. *avium* ATCC 25291.

exposed to *M. avium* subsp. *paratuberculosis* in comparison to unexposed healthy controls.

## MATERIALS AND METHODS

**Antigens.** Ten recombinant *M. avium* subsp. *paratuberculosis* antigens were used to stimulate lymphoproliferation and IFN-γ production (Table 1). Antigens were selected based on their upregulated transcriptomic (18) and proteomic (15, 16) responses to physiological stress conditions and *in silico* epitope prediction analysis (19). Two *M. avium* subsp. *paratuberculosis* antigens were produced as His-tagged recombinant proteins at the Faculty of Veterinary Science, University of Sydney, Sydney, Australia, as previously described (20). Briefly, gene-specific primers were designed to include an *att*B1 site and *att*B2 site at the 5′ end of each sequence. The complete open reading frames of each gene were amplified by PCR using Gateway Technology (Invitrogen, Australia). Amplified and purified PCR products were cloned into donor vector pDONR221 (Invitrogen, Australia) and transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Australia) to produce the entry clone. Purified entry clones were subcloned into destination vector pET160-DEST with an N-terminal 6×His and Lumio tag (Champion pET160 Gateway expression kit with Lumio technology) (Invitrogen, Australia) and transformed into One Shot TOP10 chemically competent *E. coli* cells to produce an expression clone. One Shot BL21 Star(DE3) cells (Invitrogen, Australia) were transformed with purified expression clone. The transformed culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside to express recombinant proteins. Recombinant proteins were extracted and purified by affinity liquid chromatography (AKTA purifier system; GE Healthcare). The purified recombinant protein yield was determined by a NanoDrop spectrophotometer set at an absorbance of 280 nm (Thermo Scientific).

Eight proteins were produced as maltose binding protein (MBP)-recombinant fusion proteins at the USDA ARS National Animal Disease Centre, Ames, IA, as previously described (21, 22) (Table 1). Briefly, the full-length coding sequence of the *M. avium* subsp. *paratuberculosis* protein was amplified using gene-specific primers and cloned into the pMAL-c2 translational fusion expression vector. The vector and amplified products were digested with XbaI and HindIII, and the ligated products were transformed into *E. coli* DH5α cells. The overexpressed proteins were extracted and purified by affinity chromatography with amylose resin (New England BioLabs) columns. The purified MBP-fusion protein yield was determined by a NanoDrop spectrophotometer set at an absorbance of 280 nm.

MAP 316v, a French-pressed *M. avium* subsp. *paratuberculosis* antigen produced from the laboratory-passaged isolate 316v (a generous gift from the Elizabeth Macarthur Agricultural Institute, New South Wales, Australia), was used for *M. avium* subsp. *paratuberculosis*-specific lymphocyte proliferation and whole-blood stimulation for IFN-γ induction. Other mycobacterial antigens, comprising purified protein derivatives from *Mycobacterium avium* (PPD-A) and *Mycobacterium bovis* (PPD-B) (Prionics) and a nonspecific pokeweed mitogen (PWM) (Sigma) were used as positive controls to induce whole-blood IFN-γ induction.

**Animals and blood sampling.** Blood samples were collected from Merino sheep from an ongoing experimental infection trial at the Faculty of Veterinary Science, University of Sydney. The experimental infection trial was approved by the University of Sydney Animal Ethics Committee.

A total of 33 animals broadly categorized as unexposed or exposed to *M. avium* subsp. *paratuberculosis* were included in the study. The unexposed animals ($n = 5$) were healthy controls. *M. avium* subsp. *paratuberculosis*-exposed animals were subcategorized as follows: (i) vaccinated ($n = 7$) with commercial Gudair vaccine (Pfizer Animal Health); (ii) orally challenged ($n = 11$) with three doses of 10 ml of inoculum as previously described (23), with each animal receiving $2.1 \times 10^9$ viable *M. avium* subsp. *paratuberculosis* cells (sheep strain Telford 9.2), and (iii) vaccinated with Gudair vaccine (cattle strain 316v) followed by oral challenge ($n = 10$) as described above. Animals were 4 months of age when vaccinated and 5 months of age when orally challenged (Fig. 1). All unexposed and exposed animals were managed on separate paddocks under conventional Australian sheep farming conditions by grazing in open paddocks. Vaccinated animals were given 1 ml of the required vaccine formulation by subcutaneous injection behind the ear on the upper neck region.

Blood samples (20 ml) were collected by venipuncture into lithium-heparin tubes at 8 months of age or 3 months post-oral challenge (Fig. 1) and 4 months post-Gudair vaccination. One tube of blood each was used for the IFN-γ and proliferation assays.

**IFN-γ assay. (i) Whole-blood stimulation.** Heparinized blood (500 μl per well) was placed in a 48-well plate (Falcon) and stimulated with 500 μl of antigen at a final concentration of 10 μg/ml of MAP 316v, PPD-A, or PPD-B, 5 μg/ml of PWM, 10 μg/ml of His-tagged recombinant antigen, or 20 μg/ml of MBP-fusion recombinant antigen. All antigens were diluted in culture medium (RPMI 1640, 10% [vol/vol] fetal calf serum, penicillin, streptomycin, β-mercaptoethanol, L-glutamine) (GIBCO, Life Technologies). The negative control consisted of culture medium alone. The plate was incubated at 37°C in air supplemented with 5% $CO_2$ for 48 h, as described previously (24). The plasma supernatant was then harvested and stored at −20°C.

**(ii) IFN-γ ELISA.** The IFN-γ enzyme-linked immunosorbent assay (ELISA) was performed as previously described (25, 26). A 96-well

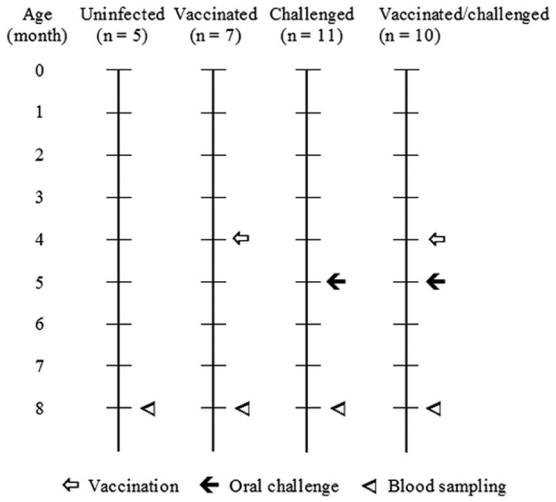


**FIG 1** Experimental timeline. The timeline includes exposure (vaccination with Gudair vaccine and oral challenge with cultured clonal inoculum of *M. avium* subsp. *paratuberculosis*) and blood sampling. The light arrow, dark arrow, and arrowhead indicate the time points of vaccination, oral challenge, and blood sampling, respectively.

MaxiSorp microplate (Nunc, Denmark) was coated with mouse monoclonal anti-bovine IFN-γ antibody (IFN 6.19) (50 μl/well), generously provided by Gregers Jungersen (National Veterinary Institute, Denmark), at a final concentration of 1.5 μg/ml in phosphate-buffered saline (PBS). The plate was incubated overnight at 4°C and then washed five times with reverse osmosis water containing 0.05% (vol/vol) Tween 20 (RO Tween water), using a 96-well plate washer (Tecan Trading AG, Switzerland). PBS (50 μl) and undiluted thawed plasma supernatant (50 μl) were added per well, and the plate was incubated for 1 h at room temperature (RT). The plate was washed as described above, and 50 μl of secondary antibody (biotin-conjugated mouse monoclonal anti-IFN-γ antibody clone CC302) (ABD Serotec, Oxford, United Kingdom) was added at a 0.5-μg/ml concentration in PBS. The plate was incubated for 1 h at RT. The plate was washed as described above, and 50 μl/well of horseradish peroxidase-streptavidin was added at a 0.01-μg/ml concentration in PBS. The plate was incubated for 1 h at RT and washed as described above. A 100-μl aliquot of substrate 3′,3′, 5,5′-tetramethylbenzidine (Thermo Fisher Scientific, Rockford, IL) was added to all of the required wells, and the mixture was incubated in the dark for 30 min. The enzymatic reaction was stopped by adding 50 μl of 2 M sulfuric acid, and absorbance was measured at the 450-nm wavelength using a plate reader (Multiskan Ascent, ThermoElectron, Waltham, MA). Serum supernatants obtained from blood samples stimulated by MAP 316v antigens were used as known IFN-γ positive and negative plate controls. The response in culture medium was subtracted from the response to each recombinant antigen to obtain the antigen-specific IFN-γ response. The IFN-γ response in stimulated whole blood was presented as an sample-to-positive (SP) ratio using the following calculation (where OD is optical density): SP ratio = (mean sample OD – mean negative control OD)/(mean positive control OD – mean negative control OD).

**Proliferation assay. (i) Isolation of PBMCs.** Heparinized blood was used to isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation as previously described (9). Briefly, blood was centrifuged at 1,455 × *g* for 20 min at RT, and the buffy coat was collected with a sterile pipette, diluted 1:3 in PBS, layered over Ficoll-Paque Plus (Pharmacia) (1:2), and centrifuged at 754 × *g* for 30 min with no braking at RT. The cloudy layer of interface containing PBMCs was aspirated with a sterile pipette and washed in PBS (30 ml) to remove platelets by centrifugation at 233 × *g* for 10 min at RT. The supernatant was discarded, and the pellet was resuspended in 30 ml PBS. Cell concentration was determined using a handheld automated cell counter (Sceptre, EMD; Millipore, Billerica, MA).

**(ii) Fluorescent labeling and cell culture.** The PBMCs were labeled with the fluorescent tracking dye carboxy fluorescein diacetate succinimidyl ester (CFSE) (Fluka, St. Louis, MO) as previously described (27). Briefly, wash buffer (5% fetal calf serum in PBS) was used to obtain a concentration of $10 \times 10^6$ cells/ml. The cells were labeled by adding CFSE to a final concentration of 5 μM and incubating the mixture in the dark for 5 min. The labeling was stopped by adding a 10× volume of ice-cold wash buffer. The cells were pelleted by centrifugation at 233 × *g* for 10 min at RT, and the supernatant was discarded. The pellet was resuspended in the residual volume, and 10 ml wash buffer was added. Cells were pelleted by centrifugation at 233 × *g* for 10 min, the supernatant was discarded, the cells were resuspended in the residual volume, and prewarmed culture medium was added to obtain a cell suspension of $5 \times 10^6$/ml. A total of $5 \times 10^5$ cells per well (100 μl) were added to a 96-well microplate containing 100 μl of medium alone (negative control) or 100 μl of antigen. MAP 316v antigen (10 μg/ml) was used as an *M. avium* subsp. *paratuberculosis*-specific positive control. The concentrations of recombinant antigens used were 10 and 20 μg/ml of His-tagged and MBP-fusion recombinants, respectively. The plates were then incubated for 6 days at 37°C in a 5% $CO_2$-supplemented incubator. All stimulations were performed in duplicate. Incubated plates were centrifuged at 233 × *g* for 3 min at RT. Supernatants were discarded by flicking the plates and wiping on tissue paper (Kim Wipe; Kimberley-Clark Professional, Irving, TX). The plate lids were put back on, and the cells were resuspended by brief vortexing. The fluorescent-labeled cells were fixed by adding 200 μl/well of 1% paraformaldehyde in PBS (Sigma-Aldrich, St. Louis, MO). The plate was protected from light and stored at 4°C, and data were acquired within 3 days.

**(iii) Flow cytometry and data acquisition.** The flow cytometric data were acquired on a high-throughput system on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest Pro software version 4.0 (BD Biosciences, San Jose, CA). The cells were characterized by a density plot based on forward scatter (FSC) and side scatter (SSC). Since an anti-ovine anti-CD3 antibody is not commercially available, mononuclear cells were selected based on size and granularity. This population of proliferating and nonproliferating cells was analyzed by a histogram depicting counts versus CFSE fluorescence. Nonproliferating cells are represented by a peak of high fluorescence indicating that those cells had retained the dye. Conversely, proliferating cells are represented by a peak of lower fluorescence as they lose fluorescence with every mitotic division. Cell proliferation data are presented as antigen-specific proliferation: % of antigen-specific proliferation – % of proliferation in culture medium.

**Data analysis.** Comparison of lymphoproliferative and IFN-γ responses between healthy controls and exposed groups was analyzed by analysis of variance (ANOVA) with a Bonferroni correction for multiple comparison (28). The correlation between the lymphoproliferation and IFN-γ responses in animals that were exposed either to vaccine or to the live *M. avium* subsp. *paratuberculosis* inoculum was analyzed. Statistical analysis and illustration were performed using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA).

## RESULTS

**IFN-γ induction.** Six recombinant antigens (MAP2698c, MAP3567, MAP0187c, MAP1560, MAP1588c, and MAP1589c) were found to induce detectable levels of IFN-γ secretion (Fig. 2A). The response was negligible from the other four recombinant antigens (MAP2487c, MAP3393c, MAP3268, and MAP1017c). The IFN-γ response to the majority of the antigens was stronger in the vaccinated or vaccinated and challenged groups of animals in comparison to animals that were challenged alone. The IFN-γ response within the groups showed substantial variation in OD values, indicating variation in response by individual animals. All of the healthy control animals had negligible levels of IFN-γ response.

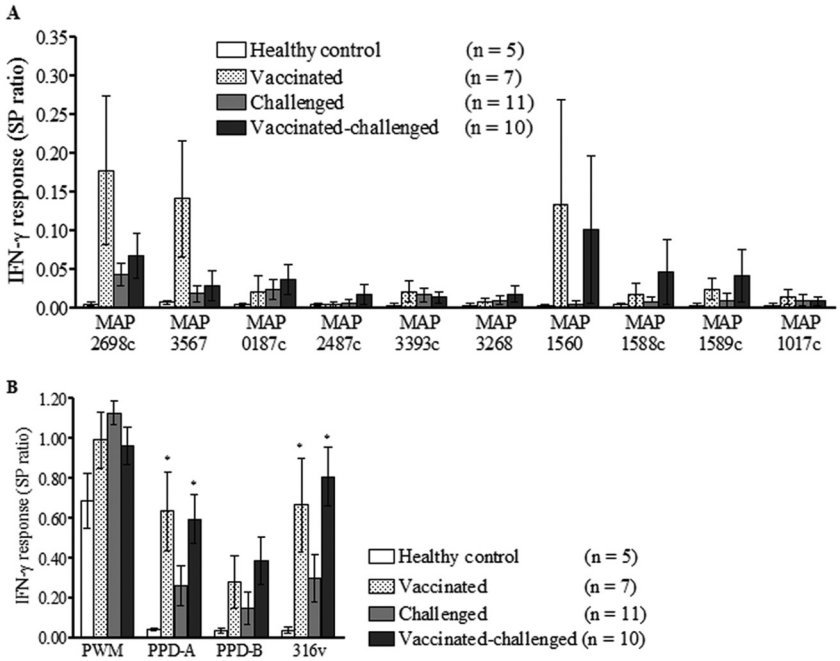


**FIG 2** IFN-γ response as recalled by recombinant proteins. Shown are the IFN-γ response to recombinant antigen-specific stimulation (A) and the IFN-γ response to nonspecific antigen (PWM), mycobacterial species-specific antigens (PPD-A and PPD-B), and MAP 316v antigen (B). The results are presented as sample-to-positive (SP) ratios. Significant differences in the mean between the healthy control and exposed (vaccinated or challenged alone and vaccinated and challenged) groups are shown by asterisks ($P < 0.05$). Error bars represent the standard errors of the SP ratio.

Whole-blood stimulation by antigens MAP1588c and MAP1589c showed higher IFN-γ responses in animals that were both vaccinated and challenged in comparison to animals that were challenged or vaccinated alone. Among all recombinant antigens, MAP2698c, MAP3567, and MAP1560 induced the greatest IFN-γ response.

The French-pressed MAP 316v antigen induced a strong *M. avium* subsp. *paratuberculosis*-specific immune memory IFN-γ recall from all three groups exposed to *M. avium* subsp. *paratuberculosis* via either vaccination or oral challenge alone or vaccination and challenge (Fig. 2B). All four groups of animals had a strong nonspecific IFN-γ response when stimulated with PWM, a nonspecific positive-control mitogen (Fig. 2B). The exposed groups of animals showed significantly higher IFN-γ responses in comparison to healthy controls when stimulated with either PPD-A or MAP 316v antigens (Fig. 2B). The PPDs derived from *M. bovis* (PPD-B) evoked weaker responses than PPD-A.

**Cell proliferation.** Figure 3 presents representative results of flow cytometry from a vaccinated sheep in response to stimulation with culture medium (Fig. 3A), test antigen (MAP2698c) (Fig. 3B), and positive-control antigen MAP 316v (Fig. 3C). After 6 days of culture, a high level of lymphoproliferation was observed to antigens MAP2698c and MAP 316v compared to culture medium alone. The proliferating cells in response to the MAP2698c and MAP 316v antigens were detected as low-fluorescence-intensity peaks of CFSE compared to a negligible amount of proliferation in culture medium (Fig. 3D).

Mean lymphoproliferation responses in each exposed group were analyzed. Although statistically not significant, the mean proliferative responses in exposed groups were empirically higher than those of the healthy control group (Fig. 4A). Within the exposed groups, three antigens (MAP2698c, MAP3567, and MAP1589c) were able to induce higher levels of lymphoproliferation in the groups vaccinated alone or vaccinated and challenged than in the group challenged alone. Antigens MAP0187c, MAP2487c, and MAP1588c induced weaker proliferation responses in the vaccinated group compared to the infected-alone or vaccinated and challenged groups. Among all antigens, four antigens (MAP2698c, MAP3567, MAP0187c, and MAP1589c) showed the highest lymphoproliferative responses.

Lymphoproliferative activity in all three exposed groups in response to the MAP 316v antigen was higher than that in the healthy control group. The response in the vaccinated group was significantly higher than that in the control group ($P < 0.05$) (Fig. 4B).

**Relationship between lymphoproliferation and IFN-γ response.** The relationship between lymphoproliferation and the IFN-γ response was examined by calculating the coefficient of correlation ($r$) in the exposed groups of animals. The responses were significantly correlated for two antigens: MAP2698c ($r = 0.53$, $P = 0.003$) and MAP3567 ($r = 0.61$, $P = 0.001$).

## DISCUSSION

This study reports the evaluation of the ability of 10 recombinant *M. avium* subsp. *paratuberculosis* antigens to evoke lymphoproliferative and IFN-γ responses in *M. avium* subsp. *paratuberculosis*-sensitized animals. Antigen-specific IFN-γ induction was measured by an IFN-γ ELISA, and lymphoproliferation was measured by flow cytometry of CFSE-labeled PBMCs.

Among the 10 antigens evaluated, antigens prepared from MAP2698c, MAP3567, MAP1589c, and MAP1560 were found to induce lymphoproliferative and IFN-γ responses in animals that

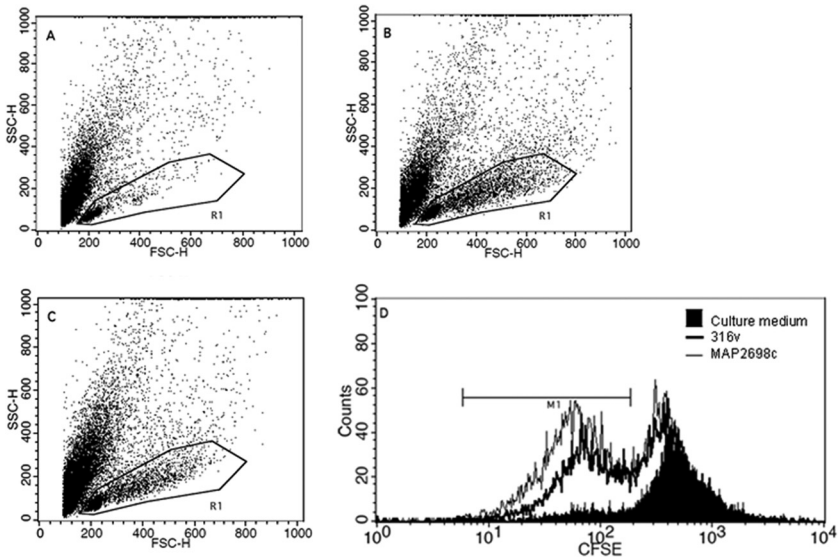


**FIG 3** Flow cytometric data of antigen-specific cell proliferation. Shown are density scatter plots of proliferating gated R1 cells (lymphocytes and monocytes) in response to stimulation by culture medium (A), recombinant MAP2698c (B), and MAP 316v antigen (C), as well as a histogram (D) of event counts against fluorescence intensity for proliferation comparison in response to culture medium, recombinant MAP2698c, and MAP 316v antigens. The M1 marker encompasses proliferating cells with lower fluorescence intensity. SSCH, side scatter; FSCH, forward scatter.

were exposed to a vaccine containing *M. avium* subsp. *paratuberculosis* immunogen and/or challenged with *M. avium* subsp. *paratuberculosis* compared to controls. *In silico* epitope prediction of MAP2698c and MAP3567 proteins found that they carry a higher number of T-cell epitopes than the other antigens examined (19). Transcriptomic studies have reported that genes encoding these four proteins are upregulated when exposed to different *in vitro* stress conditions (18, 29, 30). Furthermore, MAP2698c is an ortholog of Rv1094 of *Mycobacterium tuberculosis*, which plays an important role in the intracellular survival and growth of mycobacteria and the pathogenicity of mycobacterial infection (31). MAP3567 and MAP2698c were also reported to be recognized by serum antibody of sheep infected with *M. avium* subsp. *paratuberculosis* (20).

Lymphocyte proliferation using the MAP 316v antigen in sheep PBMCs has been reported as early as 4 months postchallenge, with peak proliferation at 8 months postchallenge (9). There were similar findings in this study using MAP 316v antigen

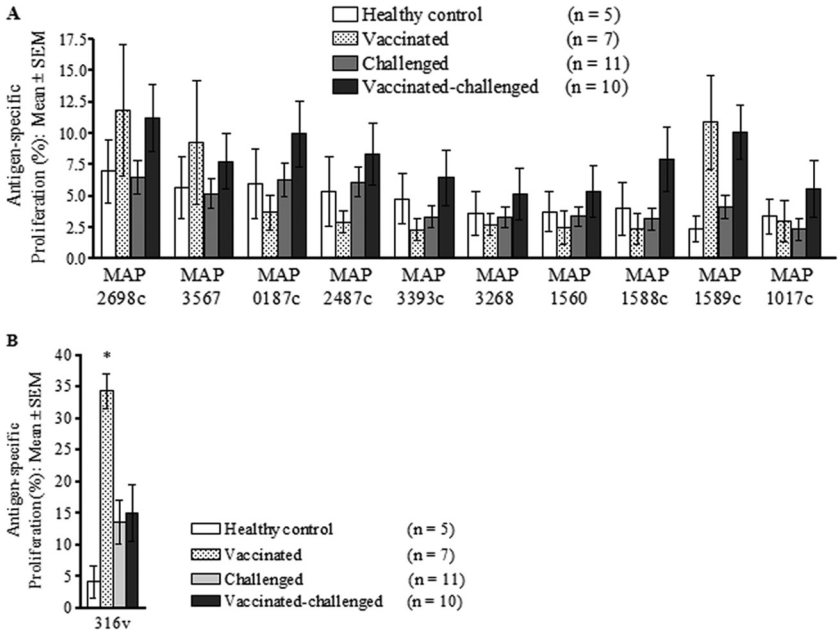


**FIG 4** Lymphoproliferative response to recombinant proteins. (A) Lymphoproliferative response to recombinant *M. avium* subsp. *paratuberculosis* antigens; (B) lymphoproliferative response to MAP 316v antigen. The results are presented as percentages of antigen-specific proliferation. Significant differences in the mean compared to the healthy control group are shown by asterisks ($P < 0.05$). Error bars represent the standard errors of the percentage of proliferation.

as a positive control. In comparison to the responses to MAP 316v and mycobacterial PPDs, the single recombinant antigen showed weaker responses of lymphoproliferation and IFN-γ secretion. However, it should be noted that the proliferative response to MAP 316v antigen is a cumulative response to the large number of proteins that it contains. It is possible that the purified antigen may not be taken up by antigen-presenting cells (APCs) in the same way as complex antigens, such as PPD or whole mycobacterial lysates, and that this may account for variation in the level of response found. However, similar studies using the purified protein ESAT-6 in the diagnosis of bovine tuberculosis have yielded robust responses (32). Another reason for the poor lymphoproliferation response may be due to the possibility of purified antigen containing endotoxins. Endotoxins may induce or be detrimental to the proliferation of PBMCs. Although the antigens used in this study were purified by affinity chromatography, and bound protein was washed with excessive amounts of buffer and then dialyzed in phosphate buffered saline, they were not tested for the presence of endotoxin.

It was disappointing to note that the lymphoproliferative and IFN-γ responses to these recombinant antigens were not significantly different from those of the control and far weaker than that of MAP 316v antigen. The findings suggest that not all *M. avium* subsp. *paratuberculosis* proteins known to be differentially regulated under stress conditions are of potential diagnostic value, as found by other studies (33, 34). There is a need to investigate other *M. avium* subsp. *paratuberculosis* proteins for diagnostic value.

The weak response of lymphoproliferation and IFN-γ secretion to recombinant antigens may also be attributed to the evaluation performed at early time points, which in this study were 3 months postchallenge and 4 months postvaccination. The responses at later time points may be stronger but were out of the scope of this study. Therefore, a longitudinal study combined with larger sample size at later time points postvaccination and/or postchallenge may provide further information on the immunogenic potential of these antigens. The level of IFN-γ produced also depends on sites of infection and polarity of disease (5, 35). High IFN-γ responses were reported in peripheral blood, gut, and prescapular lymph nodes of vaccinated animals, spleen and postjejunal lymph nodes of clinically affected animals, and only in peripheral blood of unvaccinated animals that were uninfected following experimental challenge (36, 37). The IFN-γ response measured in this study was only from peripheral blood samples.

Lymphoproliferation was found to be positively correlated to IFN-γ production. However, the correlation was weak for the majority of the proteins, which may be due to the small sample size in each group, with high variation in individual animal responses.

Lymphoproliferation and IFN-γ responses in this study were found to be by and large a vaccine effect rather than a *M. avium* subsp. *paratuberculosis* challenge effect because the response in animals that received vaccine alone and vaccine followed by *M. avium* subsp. *paratuberculosis* challenge was higher than that in the group challenged alone. This effect supports the hypothesis that live virulent *M. avium* subsp. *paratuberculosis* (oral inoculum) leads to evasion of host defense mechanisms, causing the host to fail or to delay mounting an immune response, compared to heat-killed *M. avium* subsp. *paratuberculosis* (vaccine), which is easily screened and to which the host mounts a strong immune response. This effect also reflects the potential of Gudair vaccination to induce strong cell-mediated immune responses and antibody-mediated responses, as previously reported (13).

The four recombinant antigens (MAP2698c, MAP3567, MAP1589c, and MAP1560) found to induce lymphoproliferative and IFN-γ responses in this study may need further evaluation, possibly in combination with other antigens.

## ACKNOWLEDGMENTS

This work was supported by Meat and Livestock Australia and by Cattle Council of Australia, Sheepmeat Council of Australia, and WoolProducers Australia through Animal Health Australia.

Anna Waldron and Nicole Carter of the Farm Animal Health group provided laboratory assistance in arranging ELISA reagents. Graeme Eamens, Department of Primary Industry, Elizabeth Macarthur Agricultural Institute, kindly provided access to MAP 316v antigen. Craig Kristo and Nobel Toribio provided help with animal handling and sample collection.

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