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IP10 is a predictor of successful vaccine protection against paratuberculosis infection in sheep

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ABSTRACT

The cell mediated immune response and ability of immune cells to migrate to the site of infection are both key aspects of protection against many pathogens. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is an intracellular pathogen and the causative agent of paratuberculosis, a chronic wasting disease of ruminants. Current commercial vaccines for paratuberculosis reduce the occurrence of clinical disease but not all animals are protected from infection. Therefore, there is a need to understand the immune responses triggered by these vaccines at the site of infection, in circulating immune cells and their relationships to vaccine-mediated protection. The magnitude and location of gene expression related to the cell mediated immune response and cellular migration were studied in the ileum of sheep. In addition, longitudinal IP10 (also known as IP10) secretion by circulating immune cells was examined in the same sheep. Animals were grouped based on vaccination status (vaccinated vs non-vaccinated) and MAP exposure (experimentally exposed vs unexposed). Vaccination of unexposed sheep increased the expression of *IP10*, *CCL5* and *COR1c*. Sheep that were successfully protected by vaccination (uninfected following experimental exposure) had significantly reduced expression of *IP10* in the ileum at 12 months post exposure compared to vaccine non-responders (those that became infected) and non-vaccinated infected sheep. Successfully protected sheep also had significantly increased secretion of IP10 in *in vitro* stimulated immune cells from whole blood compared to vaccine non responders at 4 months post exposure. Therefore, the IP10 recall response has the potential to be used as marker for infection status in vaccinated sheep and could be a biomarker for a DIVA test in sheep.
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1. Introduction

Paratuberculosis is a chronic enteritis of ruminants resulting in wasting and eventual death. The disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a fastidious slow growing bacterium, and is a significant financial burden to producers. Cell mediated immune responses (CMI) are required for protection against mycobacterial diseases [37]. Essential to the CMI response is the production of interferon gamma (IFNγ), activating CD4+, CD8 + and γδ T cells [10]. Interestingly, although IFNγ is produced, and is a marker of exposure, this cytokine is not predictive of paratuberculosis disease outcome or infection [19]. In vaccinated sheep, MAP-specific IFNγ responses by circulating Peripheral blood mononuclear cells can be seen in those successfully protected against infection but also in those animals that are vaccinated but succumb to the disease (vaccine non-responders) [12]. Furthermore early low IFNγ responses in sheep are predictive or more severe disease [11]. These studies suggest that, although IFNγ is important for a successful CMI response, the host response is complex and there may be other factors impeding immune protection of livestock against mycobacterial infection.

Migration of immune cells to the site of infection is crucial for an animal to mount a protective immune response. Immune cell migration or attraction to an infection site is mediated by the release of signals including chemokines and lymphokines [28]. Secretion of the chemokine IP10 (also termed CXCL10) has been shown to induce the activation and recruitment of monocytes, natural killer (NK) cells and T cells into tissue lesions in human tuberculosis and inflammatory bowel disease associated with mycobacterial infection [22,36]. In these studies, elevated levels of IP10 in the serum and secretion by re-stimulated circulating cells were associated with disease severity. Heightened *IP10* expression is also seen in serum, plasma, lymph nodes and lung granulomas of patients with active tuberculosis [1,9]. Concentra-

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tions of IP10 produced by stimulated circulating cells have also been suggested as a biomarker for active tuberculosis in cattle [16,25]. In contrast, secretion of the chemoattractant CCL5 is suppressed in circulating immune cells of cattle experimentally infected with MAP compared to control cattle [6] but was upregulated *in vitro* in a human monocytic cell line infected with MAP [35]. The differences observed in the levels of different chemoattractant suggest a possible cell- or site-specific response. Therefore, the expression levels of genes encoding the production of cytokines and other chemoattractant factors at the site of infection requires further investigation.

The ability of immune cells to migrate to, and within, the tissue is impacted by proteins within the migrating cell and surrounding cells, which can be manipulated by infectious agents. Actin binding proteins known as coronins regulate cell motility by co-ordinating actin filament turnover in lamellipodia of migrating cells [33]. The transcription of coronins can be altered by extracellular stimuli such as growth factors and bacterial products [29]. The coronin gene family includes at least six members, with *Coronin 1A* often examined in relation to bacterial diseases [14,29]. However *COR1c* plays an important role in both epithelial and fibroblast cell migration, and associated wound healing [33]. Depletion of coronin1C from cells increases migration of epithelial cells but attenuates migration of fibroblasts [30]. However the effects of *COR1c* on migration of other cell types, such as immune cells, has not been examined, and this coupled with its effect on wound healing are important factors to examine with respect to paratuberculosis infection in the gut.

Equally important to examine is the dissemination of MAP to the site of infection and how this can be influenced by or can influence the immune response. Host lymphokines such as macrophage migration inhibitory factor (MIF) impact immune cell and bacterial survival and migration [7,17,23]. MIF is released by a range of cell types in response to many different stimuli, including IFNγ, bacterial products and reactive oxygen species [39]. Depending on the cell type and environment in which it is released, MIF promotes macrophage and T cell activation, triggering both the innate and adaptive arms of the immune response. Patients with active tuberculosis have high levels of MIF in their serum [41]. This elevation was believed to be a host response to *Mycobacterium tuberculosis* associated with enhanced trafficking of macrophages to the site of infection. However, when *MIF* is expressed in microfold cells (M) in the gut, it enhances uptake and transport of these pathogenic bacteria across the intestinal barrier [23]. This increased trafficking is suggested to be a bacterial-driven process whereby phagocytosis of bacteria once they have crossed the gut barrier further increases expression of *MIF* [23]. Therefore, the location of *MIF* expression and the cell type involved greatly impacts the effect it has on the immune response of the host and the capacity to clear bacterial infection.

In order for vaccines to be protective against mycobacterial infections they should induce cell migration and a potent CMI response at the site of infection [21]. Although the above-mentioned molecules have been studied in relation to disease progression in both MAP and other mycobacterial infections, there has been little examination of the ability of vaccines against MAP to induce these responses in the gut. Therefore, the aim of this work was to assess the impact of vaccination, MAP-exposure, and the resultant disease outcome on the expression of specific genes related to cell migration and CMI response induction at the main site for MAP infection, the ileum, of sheep. For IP10, these were correlated with memory responses in circulating immune cells, indicating this is a key predictive marker of vaccine-mediated immune protection in paratuberculosis.

2. Methods

2.1. Animal experiments

Sheep used for this study were part of an two separate trials in a singular experiment utilising the same methodology and approved by the University of Sydney Animal Ethics Committee (AEC) (ref no 6064).

Sheep (Table 1.) were vaccinated with Gudair® in accordance with the manufacturer's instructions and at six weeks post-vaccination a cohort was moved to quarantine paddocks and inoculated with MAP (Telford 9.2). The inoculation consisted of 3 oral doses (within a 4-week period) as described by Begg *et al.* [2–3] for trail 1 (sheep used for gene expression) this equated to a total of 9.25×10^8 viable MAP organisms, for trial 2 (sheep used for the IP10 ELISA) this equated to a total of 2.74×10^9 viable MAP organisms [5]. A non-vaccinated cohort was similarly exposed to MAP at the same time. Unexposed animals (vaccinated and non-vaccinated), were kept in separate paddocks to their MAP exposed counterparts.

For the circulating cell stimulation assays in trial 2, blood samples were collected from sheep into lithium heparin vacuum blood tubes (Vacuette), at 4, 6 and 12 months post inoculation, and stored at room temperature for no longer than 6 h prior to performing the whole blood stimulation assay.

Animals were necropsied 12 months after exposure to MAP (13.5 months post vaccination) and multiple gut and lymph node tissues collected, as previously described [2–3]. Gene expression analysis was conducted on sections of the terminal ileum for sheep in trial 1. Infection status of all sheep across both trials was determined at necropsy by tissue culture of ileal and jejunum tissue sections to detect MAP, the gold standard for confirmation of individual animal infection; sheep that were positive by gut tissue culture were designated as infected and those that were negative were designated as uninfected [27,40].

In trial 1, 2 out of 3 of the vaccinated infected sheep had paucibacillary lesions and the third had no lesions, both of the non-vaccinated infected sheep had paucibacillary lesions. Pathological lesion type was not found to be significant.

2.2. Gene selection

The genes to be examined were selected from a normalised and statistically analysed data set generated from previous microarray gene expression analysis on sheep peripheral blood mononuclear cells (PBMC)(A. Purdie, personal communication, 2015). Ingenuity Pathway Analysis (version 01–01, Qiagen Bioinformatics) software was used to search within the data set for genes related to immune cell migration and activation of the CMI response. The overlay function was then used to examine the gene expression fold change in the microarray data set in relation to vaccination and disease outcome. Genes were then selected based on the magnitude of their fold change (greater than +/- 1.5) in PBMC for qPCR analysis in the current study (Table 2).

2.3. qPCR gene expression in sheep gut samples

At the conclusion of the animal trial the gastrointestinal tract was removed and 3–4 cm sections of the mid to terminal ileum were excised. The sections were placed in individual tubes and frozen at −80 °C pending RNA extraction.

RNA extraction was performed using RNAzol® RT (Merck) according to the manufacturer's instructions. The quality and quantity of purified RNA was assessed using a NanoDrop® ND-

Table 1
Numbers of sheep used for ViewRNA™ *in situ* and tissue qPCR gene expression analysis of the gut and IP10 ELISA.

Treatment	MAP exposure status	*MAP infection status	n for quantitative gene expression	n for *in situ* gene expression	n for IP10 ELISA
Non-vaccinated	Control	Uninfected	3	3	
	Exposed	Infected	3	3	
		Uninfected	3	1	
Vaccinated	Control	Uninfected	3	3	10
	Exposed	Infected	3	2	5
		Uninfected	3	1	15

* Infection status of the sheep was determined at necropsy (12 months post exposure) by culture of gut tissue to detect MAP.

Table 2
Primers for quantitative gene expression analysis of intestinal tissue.

Primer name	Primer sequence	Tm	Product size (bp)
COR1c	5′- CCAGGCTCAGAGTTGGTGTT-3′	59.9	547
	5′- CGACAGAATACGATGGCTGC-3′	59.1	
CCL5	5′- CGCCAACCCAGAGAAGAAGT-3′	60.0	416
	5′- GAGGGTCTTTCACAGCAGCT-3′	60.0	
IP10	5′- GCTACTGACAGTTTCCTCCCC-3′	60.1	486
	5′- AGAATATGGGCCCCTTGGAG-3′	58.9	
MIF	5′-GCAAGCCGGCACAGTACAT-3′	61.0	305
	5′-ATGTAGATCCTGTCCGGGCT-3′	60.1	
Reference gene			
Ovine GAPDH	AGAAACCTGCCAAGTATGATG	60.5	76
	CCTAGAATGCCCTTGAGAGG	62.6	

1000 UV–Vis Spectrophotometer (Thermo Scientific, Wilmington, DE), using the Nucleic Acid module. To increase RNA purity the samples were DNase treated and ethanol precipitated following extraction. cDNA was generated from RNA using the iScript™ cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions, diluted 1/10, dispensed in single-use aliquots (to avoid freeze-thawing damage) and stored at −80 °C until required.

2.3.1. Primer selection and validation

Forward and reverse primers (Table 2) were designed specifically for the gene regions of interest using online software Primer 3 [31], and checked for specificity using a BLAST search. As genes were selected based on expression levels in the PBMC, gene expression in intestinal tissues was confirmed using cDNA generated from a paratuberculosis infected sheep. Three additional previously validated housekeeping genes were assessed with geNorm analysis in the qBASE plus analysis software (Biogazelle) (data not shown) [38]. The analysis identified the most stable reference gene was GAPDH. For the remainder of the analysis, one reference gene was used based on the geNorm analysis.

2.3.2. qPCR and expression level analysis

qPCR was performed using an Mx3000P Real-time PCR system (Stratagene, Agilent) using the SensiMix™ SYBR® kit (Bioline). Assays were prepared in 96-well plates and included duplicates of each sample. Reaction volumes of 25 µL (including 10 µL of target cDNA at a 1/10 dilution) were prepared and amplified under the following conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 20 s, 56 °C for 30 s and 72 °C for 30 s, with fluorescence acquisition at the end of each annealing step. The specificity of the reaction was confirmed using melt curve analysis. Standard curves were performed on each plate for each primer set, and each sample type. Data collected from the qPCR were analysed using a modified delta delta Ct approach, the common base method [15]. Fold changes were determined using each experimental group as a comparison (control) group to understand the pairwise interactions of gene expression and vaccination status coupled with disease outcome.

2.4. *ViewRNA™ in situ gene expression in sheep gut samples*

At necropsy, sections of the mid to terminal ileum were embedded into Tissue-Tek® O.C.T compound (Sakura) in a cryomould. The cryomould was then placed into a metal beaker containing 2-methylbutane which was kept cold. After the sections were completely frozen and the OCT was opaque, cryomoulds were stored at −80 °C. The embedded tissues were sectioned at 5 µm on a cryostat (Leica 1510S) and mounted on Superfrost Plus™ adhesions slides (Mendel Glaser). Sections from two animals were mounted per slide. A subset of the sheep were chosen for qPCR gene expression were based on the quality of the tissue sections for *in situ* gene expression (Table 1).

Staining of tissue sections to visualise *in situ* gene expression was by ViewRNA™ ISH Tissue 2-Plex Assay (Affymetrix) according to the manufacturer's instructions, following the modified protocol for frozen tissues. Probes were allocated for the *in-situ* gene expression analysis so that two genes could be examined per tissue section (Table 3).

2.4.1. Fluorescence image capture

The labelled sections were examined, and images captured at 10x magnification using a Mantra Quantitative Pathology Imaging System (PerkinElmer). The system consists of the Mantra instrument, a customized research grade Olympus microscope, a multispectral 12-bit camera and 5 epi-fluorescence filter cubes (DAPI, FITC, TRITC, Cy5 and Cy7). Exposure duration was determined for each filter cube to ensure the optimal intensity was captured. All available spectra were captured for each tissue section (Table 4).

2.4.2. Image J processing and analysis

Image analysis was conducted using Fiji software (ImageJ) [34]. The stacked image for each filter cube was unstacked and the wavelength for peak dye emission was selected (Table 4). The selected images were merged into a composite and pseudocolour was added. Location of gene expression was determined in relation to the major structures of the ileal matrix (Fig. 1.) A qualitative analysis was performed to assess differences in the location of gene expression in relation to vaccination and exposure to MAP.

2.5. Whole blood stimulation assay

The whole blood stimulation assay was carried out as previously described [5]. In summary, 0.5 ml of heparinised blood was

Table 3
Probe allocation for *in situ* gene expression analysis.

Gene Name	Affymetrix probe ID	Accession number	Probe type
MIF	Bt.15528.1.S1_at	NM_001033608	1
COR1c	Bt.656.2.S1_at	NM_001081590	6
CCL5	Bt.19462.1.A1_at	NM_175827	1
IP10	Bt.16966.1.S1_at	NM_001046551	6

Table 4
Filter cube information for the Mantra Quantitative Pathology Imaging System.

Filter cube		Wavelengths captured (nm)
DAPI	Emission: Semrock FF02-409/LP-25 Excitation: Semrock FF01-387/11–25	440*, 460, 480, 500, 520, 540, 560, 580 and 600
FITC	Emission: Chroma ET510LP Excitation: Chroma ET480/40x	520, 540, 560, 580*, 600, 620, 640, 660 and 680
TRITC	Emission: Chroma HQ572LP Excitation: Chroma HQ545/30x	570, 590, 610, 630, 650, 670 and 690
Cy5	Emission: Chroma ET700/75 *m* Excitation: Chroma ET620/60x	680, 700 and 720*
Cy7	Emission: Semrock FF02-809/81–25 Excitation: Semrock FF01-708/75–25	700

* wavelengths chosen for each fluorophore to examine (DAPI = 440 nm, Fast Red = 580 nm and Fast Blue = 720 nm).

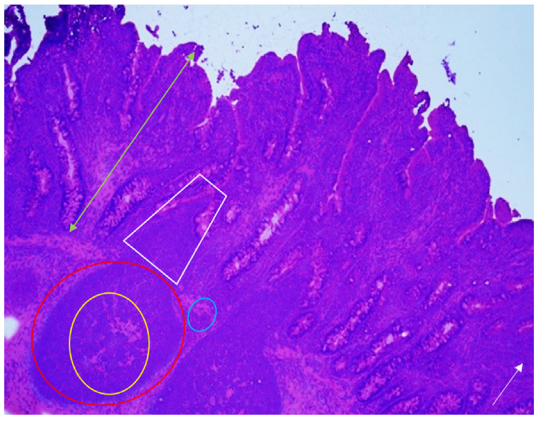

Fig. 1. Morphology and terminology used to describe gene expression location within the ileum of sheep. The structure of the ileal mucosa is shown with a haematoxylin and eosin stain. The structures shown are the dome (white line), follicle (red line), germinal centre (yellow line) all of which make up the Peyer's patch, and the interfollicular T cell zone (blue line). The lamina propria (green arrow) extends from the villus tip to the muscularis mucosa. The white arrow denotes image orientation with its point directed towards the lumen.

placed into wells of a 48-well plate with 0.5 ml culture media (RPMI 1640 (Gibco BRL) supplemented with 10% foetal calf serum (Gibco BRL), L-glutamine and penicillin/streptomycin (Gibco BRL). Samples were stimulated with MAP 316v antigen (EMAI Australia) at a final concentration of 20 μg/ml. The negative control consisted of 0.5 ml culture media and whole blood. The positive control was pokeweed mitogen (PWM (Sigma-Aldrich)) at a final concentration of 10 μg/ml in. The samples were incubated at 37 °C in air supplemented with 5% CO2; after 48 h of culture, the plasma supernatant was collected and stored at −20 °C prior to use in the IP10 ELISA.

2.5.1. Optimised IP-10 ELISA protocol

The IP-10 levels in stimulated whole blood samples were measured using the optimised protocol for the ovine *IP10* do-it-yourself ELISA (Kingfisher Biotech). Nunc Maxisorb microtitre plates (96-well) (Sigma Aldrich) were coated with anti-bovine *IP10* polyclonal antibody (1 μg/ml; 50 μL/well) in PBS and incubated overnight at 4 °C. Wells were washed 4 times between all subsequent steps with PBS/0.05% Tween 20 (300 μL/well/wash) and subsequent reactions were performed at room temperature. Samples and controls were added in duplicate diluted 1:2 in PBS (100 μL/well). Samples consisted of plasma supernatant from MAP 316v antigen-stimulated whole blood, PWM stimulated and unstimulated whole blood (control). Recombinant ovine IP10 standards in PBS were added to each plate at a final concentration of 1250–80,000 pg/ml. Plates were incubated for two hours and then were washed. Biotinylated anti-bovine IP10 polyclonal antibody (0.5 μg/ml; 50 μL/well) was added and incubated for 1 h. Subsequently, HRP-Streptavidin (1:20,000; 50 μL/well) (Thermofisher Scientific) was added and incubated at room temperature for 30 min. Following a final wash step, TMB (100 μL/well) (Thermofisher Scientific) was added and plates incubated 20 min in the dark. The reaction was stopped with 2 M sulfuric acid (100 μL/well) and the OD was read at 450 nm using an ELISA microplate reader (Multiskan Ascent, Thermo Electric Corporation).

Plasma IP-10 concentrations of samples were calculated by converting the raw OD values to SP% (per the equation below) to normalise between plates. The IP10 concentration was then determined by interpolation (logarithmic relationship) using a recombinant ovine IP10 standard curve, included in each experiment.

$$SP\% = \frac{OD_{sample} - OD_{negativecontrol}}{OD_{positivecontrol} - OD_{negativecontrol}} \times 100$$

2.6. Statistical analysis

For gene expression data, a *t*-test was performed to determine differences, using the normalised Cq values from the common base method, as reported by Ganger et al. [15]. A p value of<0.05 was considered statistically significant.

Restricted maximum likelihood (REML) in a linear mixed model (Genstat 18th edition; VSN International Ltd., Hemel Hempstead, United Kingdom) was used to analyse the IP10 ELISA results.

Sheep were grouped based on treatment coupled with infection status (vaccinated control, vaccinated infected and vaccinated uninfected) which along with sampling time point was included as a fixed effect in the model. Animal ear tag number was included as a random effect. When the REML analysis was significant, post-hoc tests to determine the significant differences between pairs of predicted means using the Fisher's Least Significant Difference (LSD) procedure were performed.

3. Results

Analysis of sections of sheep ileal tissue was conducted to assess expression of *CCL5*, *IP10, MIF* and *COR1*cat the predilection site for paratuberculosis infection. Fold changes were determined with a control group to establish pairwise comparison.

3.1. Gene expression changes in the ileum in sheep vaccinated against MAP

In sheep that were not exposed to MAP, there were no significant differences in the expression of the four candidate genes in

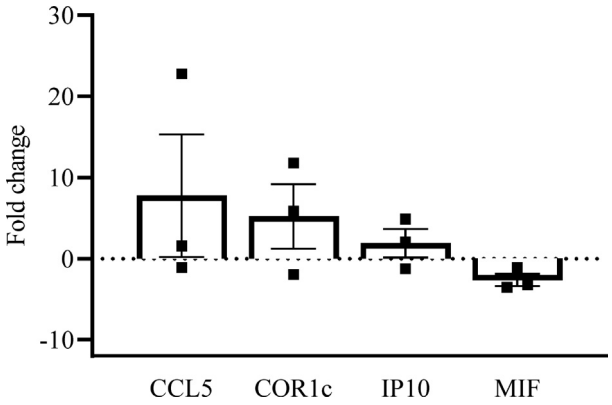

Fig. 2. Expression of *IP10, CCL5, COR1c* and *MIF* in the ileum of vaccinated control sheep that were not exposed to MAP. Gene expression in the ileum (13 months post vaccination) was examined in sheep not exposed to MAP vaccinated with Gudair® in comparison to non-vaccinated control sheep. Expression levels of *IP10*, *CCL5*, *COR1c* and *MIF* were determined by qPCR and common base method analysis. Individual animal fold change values, the mean fold change (bar) and standard error of the mean are shown in comparison with non-vaccinated, aged-matched sheep.

the ileum of sheep vaccinated with Gudair (vaccinated controls) compared to non-vaccinated sheep at 13 months post vaccination (Fig. 2.(Supplementary table 1).

The *in situ* staining location of *CCL5* expression in non-vaccinated control and vaccinated control animals (Fig. 3.) was similar, with expression in both groups spread throughout the lamina propria (LP). However, there was a difference in the pattern of staining: in the non-vaccinated controls, discrete cellular structures were observed dispersed throughout the LP, whereas in the vaccinated control sheep the staining filled all of the LP and the high intensity rendered the colouring yellow instead of green when the image was captured. The staining location of *COR1c* was almost exclusively in the Peyer's Patches (PP), with more discreet cellular structures seen in the serosal end of the PP of vaccinated controls compared to the non-vaccinated controls. The expression location for *IP10* was similar to *CCL5*. There was more *IP10* expression in the vaccinates where discrete red structures were seen spread throughout the LP. In the non-vaccinated controls, the small amount of *IP10* staining was localised to the submucosa, and was mostly in the interfollicular T cell zone, alongside the PP. Lastly, *MIF* expression was predominantly localised to the epithelium of the villi and the dome of the PP. Non-vaccinated control sheep had strong expression of *MIF* in the cells lining the dome of the PP, presumably M cells. There was also some expression in the epithelial cells of the villi. Vaccinated control animals had very similar location of *MIF* staining, in the epithelial cells and M cells of the PP however it also appeared to be spread throughout the villi, as in the non-vaccinated sheep.

3.2. Gene expression changes in the ileum in vaccinated sheep exposed to MAP

There were no significant differences in the expression of the four candidate genes examined by qPCR in in the ileum of vaccinated sheep exposed to MAP compared to vaccinated unexposed (control) sheep at 13 months post vaccination (Fig. 4.) (Supplementary table 1). However there was a trend that there was a decreased in expression of *CCL5*, *COR1c* and *IP10* in the vaccinated exposed sheep compared to the vaccinated control sheep.

3.3. Gene expression changes in the ileum in non-vaccinated sheep exposed to MAP

There were no significant differences seen in the expression of any of the genes examined by qPCR in non-vaccinated sheep exposed to MAP compared to non-vaccinated unexposed (control) sheep in the ileum at 12 months post exposure (Fig. 5.) (Supplementary table 1). However for three genes (*CCL5*, *COR1c* and *MIF*) there was a trend towards an decrease in expression in the non-vaccinated exposed sheep compared to the non-vaccinated control sheep.

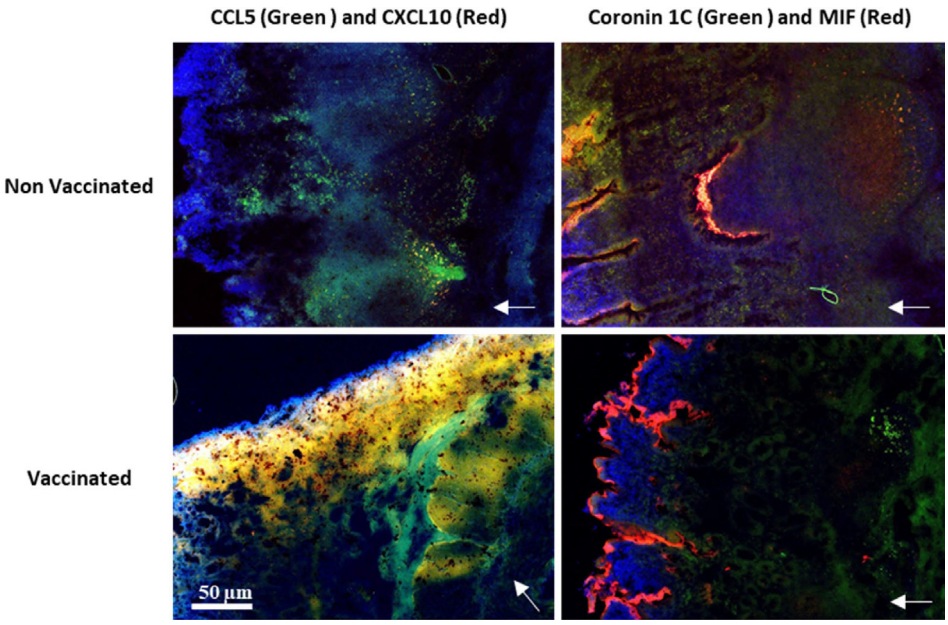

Fig. 3. Composite images of gene expression staining in the ileum of vaccinated and non-vaccinated control sheep. Gudair™ vaccinated (6 weeks prior to exposure) (n = 3) and non-vaccinated sheep (n = 3) were necropsied 13 months after vaccination and sections of the terminal ileum used for ViewRNA™ *in situ* gene expression analysis. Representative images are shown from each group. The white arrow denotes image orientation pointing towards the villi. Yellow staining shows areas of high intensity green staining that was captured as yellow at this magnification.

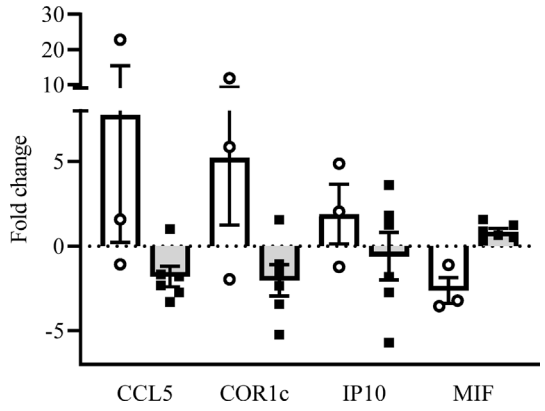

Fig. 4. Expression of *IP10, CCL5, COR1c* and *MIF* in the ileum of vaccinated sheep exposed to *Mycobacterium avium* subsp. *paratuberculosis*. Gudair® vaccinated sheep were either exposed (6 weeks post vaccination) or left unexposed (control) to MAP. At necropsy (13 months post vaccination), expression levels of *IP10, CCL5, COR1c* and *MIF* were determined in the ileum by qPCR and common base method analysis. Individual animal fold change values and the mean fold change (bar) are shown for vaccinated exposed (grey bars with filled icons) in comparison to aged-matched vaccinated unexposed (open bar with open icons).

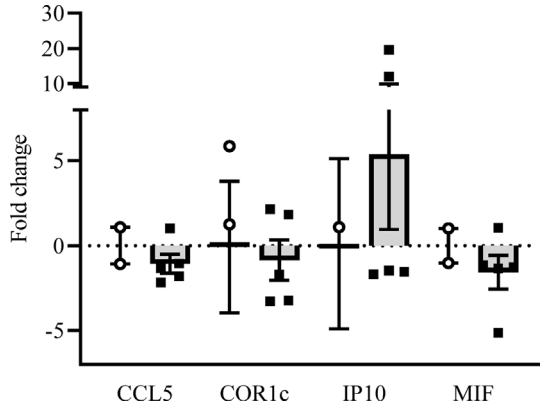

Fig. 5. Expression of *IP10, CCL5, COR1c* and *MIF* in the ileum of non-vaccinated sheep. Non-vaccinated sheep were either exposed or left unexposed (control) to MAP. At necropsy (12 months post exposure), expression levels of *IP10, CCL5, COR1c* and *MIF* were determined in the ileum by qPCR and common base method analysis. Individual animal fold change values and the mean fold change (bar) are shown for non-vaccinated exposed sheep (grey bars with filled icons) in comparison to aged-matched non-vaccinated control sheep (open bar with open icons).

3.4. Impacts of disease outcome on gene expression in the ileum of sheep exposed to MAP

Significant differences were found in the expression of *COR1c*, *IP10* and *MIF* in the ileum of both vaccinated and non-vaccinated sheep based on to disease outcome at necropsy (infected vs uninfected) (Fig. 6.). However, there were no significant differences between groups for the expression of *CCL5*.

Expression levels of both *IP10* were significantly elevated in vaccinated ($p = 0.017$) and non-vaccinated ($p = 0.003$) infected sheep compared to vaccinated uninfected sheep (Fig. 6B.) (supplementary Table 1). Expression of *MIF* was significantly increased in the ileum of vaccinated infected sheep compared with vaccinated uninfected ($p = 0.028$) (Fig. 6C.). Lastly, a significant increase in the expression of *COR1c* in non-vaccinated infected sheep compared to vaccinated uninfected sheep ($p = 0.035$) (Fig. 6A.) (supplementary Table 1) was also found in the terminal ileum at 13 months post vaccination.

Although the expression level of *CCL5* in vaccinated and non-vaccinated sheep with different disease outcome was not found to be significantly different, expression location was observed to be different depending on infection status. The non-vaccinated uninfected animals had minimal staining, which was seen in some parts of the LP and the submucosa (Fig. 7A). In contrast, the non-vaccinated infected sheep had expression of *CCL5* in the LP and the PP (Fig. 7C). The vaccinated uninfected animals had expression localised to the LP, particularly close to the muscularis mucosa (Fig. 7E). The vaccinated infected animals had minimal *CCL5* fluorescence expression, which was strongest in the PP, with some staining in the LP as well (Fig. 7G).

Expression of *COR1c* was only found in the PP across all groups of sheep except non-vaccinated control sheep and vaccinated uninfected. The non-vaccinated MAP exposed but uninfected sheep had expression of *COR1c* in the PP located in the periphery of the follicle (Fig. 7B). Non-vaccinated infected sheep also had expression in the PP, but this was found to surround the germinal centre (GC) (Fig. 7D). Vaccinated uninfected sheep had faint *COR1c* staining in the LP (Fig. 7F), whereases the vaccinated infect sheep had strong expression particularly in the serosal end of the PP (Fig. 7H).

The expression location for *IP10* was similar to *CCL5*. In non-vaccinated exposed animals, minimal staining was seen in the uninfected sheep, with only a few small pockets of *IP10* expression in the PP (Fig. 7A). In contrast in the non-vaccinated infected animals, there was strong *IP10* expression localised to the PP (Fig. 7C). The staining in the PP was found in the periphery, and towards the serosal end. A similar pattern of expression was seen in the vaccinated exposed sheep. The vaccinated uninfected sheep had no visible *IP10* staining in any of the animals assessed (Fig. 7F). In contrast, the vaccinated infected animals had stronger expression of *IP10,* which was located in the submucosa and the PP. The staining seemed to be in aggregates, presumably where there are groups of activated cells (Fig. 7G).

MIF expression was predominantly localised to the epithelium of the villi and the dome of the PP. In the non-vaccinated infected animals, expression of *MIF* could be seen in the crypts of the villi (Fig. 7D). Interestingly the same patterns were also seen in the vaccinated exposed sheep regardless of disease outcome (Fig. 7F,H).

3.5. Impacts of disease outcome in vaccinated sheep on IP10 secretion by circulating immune cells

Significant differences in IP-10 recall responses were observed ($p < 0.05$) between infected and uninfected vaccinated exposed sheep at 4- and 6-months PI (Fig. 8.). Stimulated cells from vaccinated exposed infected sheep produced significantly lower amounts of IP10 compared to vaccinated exposed uninfected sheep at 4 months post inoculation ($p = 0.019$). In contrast by 6 months post inoculation stimulated cells from vaccinated exposed infected sheep produced significantly greater amounts of IP10 compared with vaccinated exposed uninfected sheep ($p = 0.005$).

4. Discussion

In this study, we have shown that protection against ovine paratuberculosis is associated with reduced expression of *IP10* in the ileum of sheep at 12 months post exposure to MAP. In infected exposed sheep, both vaccinated and non-vaccinated, the expression of *IP10* was significantly increased compared to vaccinated sheep that were uninfected at the end of the trial. Strikingly, differences between infected and uninfected sheep seen at the level of gene expression in the ileum were also seen in the results from stimulation of circulating cells. Stimulation of circulating cells from vaccinated exposed uninfected sheep induced a greater production

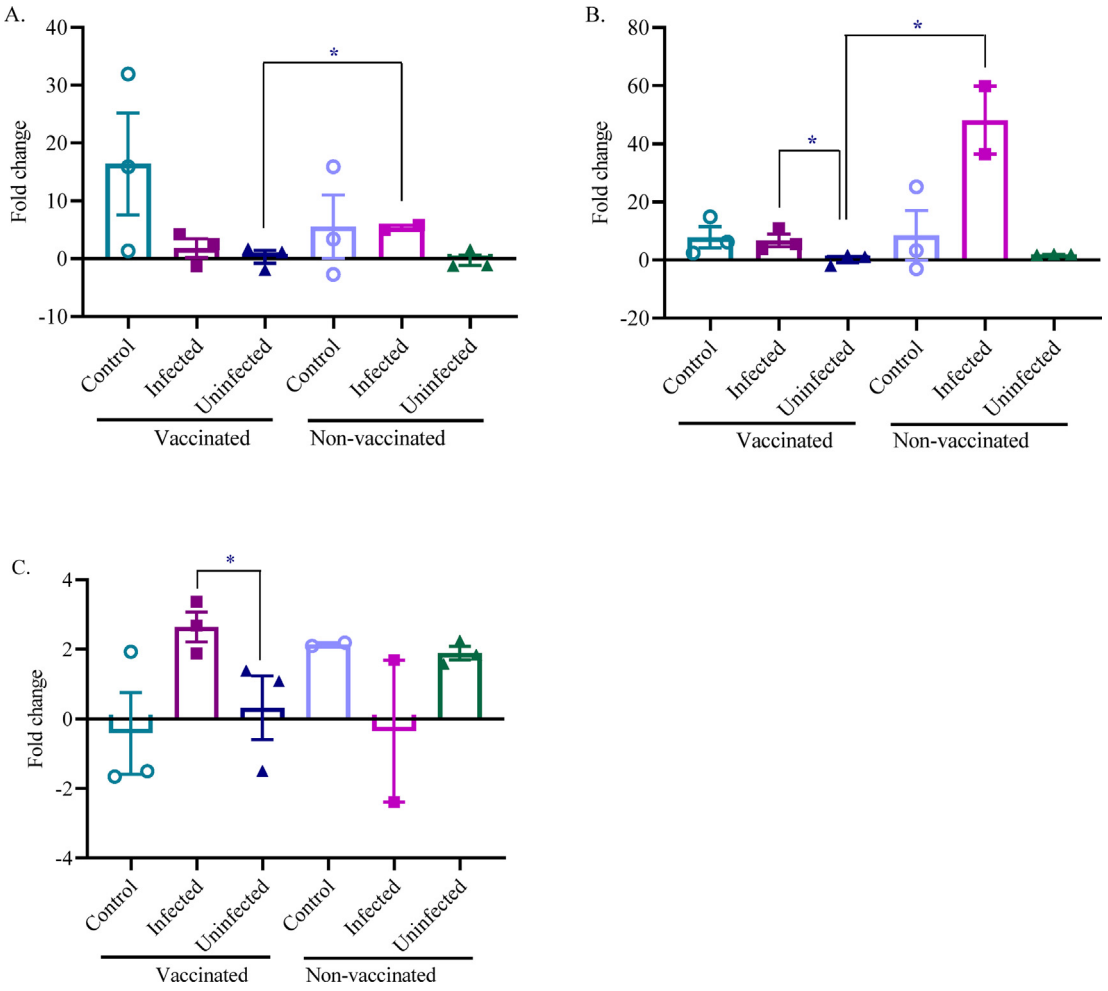

Fig. 6. Expression of *COR1c* (A), *IP10* (B) and *MIF* (C) in the ileum of sheep based on disease outcome. Gudair® vaccinated (6 weeks prior to exposure) and non-vaccinated sheep were either exposed or left unexposed (control) to MAP. Expression levels of *COR1c*, *IP10* and *MIF* were determined in the ileum by qPCR and common base method analysis. Individual animal fold change values, the mean fold change (bar) and standard error of the mean are shown are in comparison with vaccinated uninfected sheep and * denotes a statistically significant difference between two groups ($p < 0.05$).

of IP10 at 4 months post MAP exposure compared to vaccinated exposed infected cells. Interestingly, the inverse was true at 6 months post exposure, whereby the vaccinated exposed infected animals showed significantly elevated levels of secretion of IP10 from stimulated circulating cells compared to the vaccinated exposed uninfected group, which mirrored the results seen in the ileum of these animals at necropsy. Further examination of the gene expression in the ileum of infected and uninfected animals at earlier timepoints post exposure, particularly 4 months, would be required to confirm the mirroring of these responses.

Elevated expression of *IP10* (or IP-10) in circulating immune cells is well documented as a marker for active tuberculosis infection and agrees with our results [1,22,32]. A study by Marfell et al. [24] has also shown that macrophages from red deer susceptible to MAP infection have increased *IP10* expression after *in vitro* exposure. This chemoattractant has an important role in the recruitment of T cells to the site of inflammation and as such has been identified as a promising biomarker for determining active and latent tuberculosis infection, commonly utilised in conjunction with IFNɣ. In our study, sheep successfully protected by vaccination (vaccinated exposed uninfected) had elevated production of IP10 by circulating immune cells which was then down regulated by 6 months, and lower expression levels in the ileum were also observed at necropsy compared to vaccinated infected sheep. Importantly, IFNɣ secretion assays for sheep are unable to differentiate between vaccinated and infected individuals. However, the production of IP10 by circulating cells and the expression of *IP10* in the ileum was significantly different in vaccinated uninfected and vaccinated infected sheep. It is likely that the differences observed in the ileum are suggestive of an active inflammatory response in the infected sheep, and increased trafficking of MAP which could have contributed to chronic infection, compared to return to a resting state or homeostasis in sheep that are uninfected or have cleared infection. Therefore, the expression of this chemoattractant at the site of infection acts as a potential marker for infection status in both vaccinated and non-vaccinated sheep. Furthermore, the correlation with the level of IP10 produced by circulating immune cells suggest this may be a useful biomarker of protective efficacy of vaccines for paratuberculosis, with the ability to distinguish vaccinated exposed infected and vaccinated exposed uninfected animals at the level of both ileal gene expression and the circulating cells. It may also be a candidate for a DIVA test, although due to the limited numbers of sheep, and the the predictive value of IP-10 was assessed in experimentally infected animals where the timing of exposure is known and synchronous there is a need to assess sheep for protective vaccination at earlier timepoints (<4 months post-exposure) and on commercial flocks to clarify the usefulness of this biomarker.

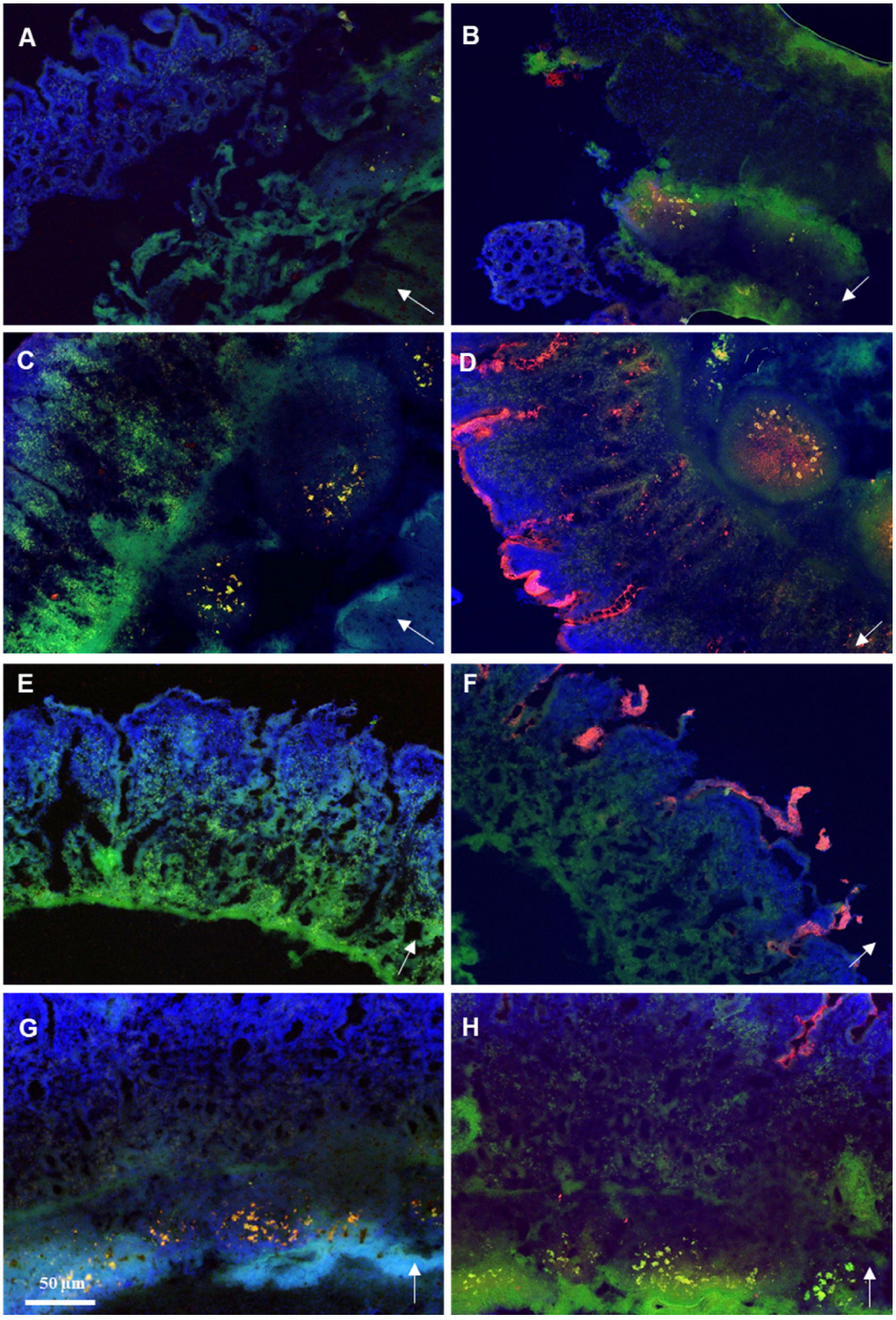

Fig. 7. Composite images of *in situ* gene expression staining in the ileum of sheep representative of disease outcome. Gudair™ vaccinated (6 weeks prior to exposure) and non-vaccinated sheep were either exposed or left unexposed (control) to MAP. Animals were necropsied 12 months after exposure and sections of the terminal ileum used for ViewRNA™ *in situ* gene expression analysis. Infection status of the sheep was determined at necropsy by culture of gut tissue. Representative images are shown from each group; (A) and (B) are non-vaccinated uninfected; (C) and (D) are non-vaccinated infected; (E) and (F) are vaccinated uninfected and (G) and (H) are vaccinated infected. Images show a composite stack of two genes of interest. Panels A, C, E and G were stained for *CCL5* (green) and *IP10* (red); panels B, D, F and H were stained for *COR1c* (green) and *MIF* (red). The white arrow denotes image orientation with the point towards the villi.

Expression of *CCL5*, *COR1c*, *IP10* and *MIF* in non-exposed sheep was increased at 13 months post Gudair™ vaccination compared to non-vaccinated sheep. *CCL5* is a potent chemokine that plays an important role in recruitment of memory T cells, monocytes and macrophages to sites of infection [6]. Through attraction of these cell types, it has been suggested that the expression of

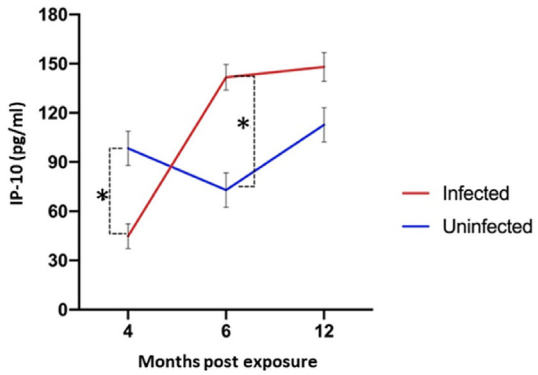

Fig. 8. Comparison of IP10 secretion by circulating immune cells in vaccinated sheep based on disease outcome. Gudair vaccinated sheep exposed to MAP had blood collected at 4, 6 and 12 months post exposure for whole blood stimulation assays. Supernatants from the assays were then examined with an IP10 ELISA to assess the IP10 recall response. Sheep were retrospectively grouped based on infection status determined by culture of gut tissue 12 months after exposure. Data shown is predicted means and standard errors. * Denotes significant differences between groups ($p < 0.05$) determined using a restricted maximum likelihood linear mixed model.

CCL5 plays an important role in granuloma formation [8,13]. In agreement with our work, vaccination of guinea pigs with BCG increased expression of *CCL5* in spleen cells [18]. Although vaccination can enhance *CCL5* expression, subsequent exposure of vaccinated animals to mycobacteria causes suppression of this gene, as seen in the vaccinated exposed cohorts [6,18,20]. It is therefore possible that these genes are markers of exposure and not influential in success of vaccine protection. *COR1c* belongs to the coronin gene family which regulates actin-dependent processes such as cell motility and vesicle trafficking [29]. Interestingly, the expression of *COR1c* in the vaccinated controls was predominantly in the serosal end of the PP. The localisation of expression to this area could promote increased motility of immune cells within the PP, allowing interaction between cell types which could facilitate activation and maturation. The increased expression of both genes in the ileum of vaccinated sheep is likely to increase immune cell migration to this site and prepare the tissue for bacterial invasion. The decreased expression of *MIF* in vaccinated control sheep is likely to reduce the transport of MAP by M cells across the intestinal border, thereby reducing infectious burden [23]. Therefore, Gudair™ vaccination of sheep augments the immune response at the site of infection and promotes a response that is likely to enhance protection against MAP invasion.

In situ gene expression visualisation in the gut of sheep correlated with quantitative gene expression results in the same tissues. Increased intensity and quantity of staining was observed in the non-vaccinated infected animals, in agreement with increased expression levels found in this group through qPCR in comparison to all other treatment groups. The localisation of expression was different dependent on vaccination status and disease outcome. The vaccinated infected sheep had strong staining in PP, whilst the uninfected sheep had staining localised to the LP. The invasion of MAP through the M cells of the PP, which in the infected animals may have been facilitated by the increased expression of *MIF*, means that bacteria are likely to reside within macrophages inside the PP, and hence the expression of genes associated with infection response appears to be localised to the infection site. These *in situ* visualisation results provide a unique understanding of the localisation of gene expression within the ileum even though sample size and selection of controls has limited the quantitative analysis of results obtained using this method.

Gene expression profiles of sheep 12 months post exposure provides insight into disease progression and vaccine elicited immune responses, but the ability to detect favourable vaccine responses earlier through production of IP10 in circulating immune cells provides an invaluable tool to producers in preventing paratuberculosis infection in their flocks. Use of the IP10 stimulation assay as an on-farm screening tool could allow detection of vaccine non-responders and early removal from the flock, as these animals are likely to perpetuate the infection cycle. The ability of vaccines to induce migration of immune cells to the site of paratuberculosis infection, is of high importance for efficacy against the pathogen. The recent discovery of the importance of tissue resident memory cells against mycobacterial diseases, only serves to emphasise the importance of assessing the ability of vaccines to induce protective types of responses [26]. Previously the response to paratuberculosis vaccination at the site of infection has been examined only in relation to the cell types present [4]. However, the genes associated with migration of these cells, and the location of their expression has not been considered. Through correlation of gene expression results at the site of infection with longitudinal assessment of IP10 production in circulating immune cells we have determined a possible biomarker of an individual animal response to vaccination and disease outcome which allow interventions to prevent spread of infection.

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Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hannah Pooley reports financial support was provided by Meat and Livestock Australia.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.11.018.

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