Indoleamine 2,3-dioxygenase (IDO), tryptophan catabolism and
Mycobacterium avium subspecies paratuberculosis: a model for chronic
mycobacterial infections.

Running title: IDO, tryptophan and host:mycobacteria interactions.

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Virulent mycobacterial infections progress slowly with a latent period that leads to clinical disease in a proportion of cases. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an intracellular pathogen that causes paratuberculosis or Johne’s disease (JD), a chronic intestinal disease of ruminants. Indoleamine 2,3-dioxygenase (IDO), an enzyme that regulates tryptophan metabolism, was originally reported to have a role in intracellular pathogen killing and has since been shown to have an important immunoregulatory role in chronic immune diseases. Here we demonstrate an association between increased IDO levels and progression to clinical mycobacterial disease in a natural host, characterizing gene expression, protein localization and functional effects. IDO mRNA levels were significantly increased in MAP-infected monocytic cells. Both IDO gene and protein expression was significantly upregulated within the affected tissues of sheep with JD, particularly at the site of primary infection, the ileum, of animals with severe multipacillary disease. Lesion severity was correlated with the level of IDO gene expression. IDO gene expression was also increased in peripheral blood cells of MAP-exposed sheep and cattle. IDO breaks down tryptophan and systemic increases were functional as shown by decreased plasma tryptophan levels, which correlated with the onset of clinical signs, a stage well known to be associated with Th1 immunosuppression. IDO may be involved in down-regulating immune responses to MAP and other virulent mycobacteria, an example whereby the pathogen may harness host immunoregulatory pathways to aid survival. These findings raise new questions about the host:mycobacteria interactions in the progression from latent to clinical disease.
INTRODUCTION

Many similarities exist between the pathogenesis of diseases due to virulent mycobacteria in man and animals. The differences mainly relate to host preference and tissue tropism, such that studies in one natural model may inform others (51). Slow progression of the disease and latent infection are hallmarks of pathogenesis and have been related to both host and microbe factors (22, 51). Paratuberculosis or Johne’s disease is a chronic infection of ruminants such as cattle, sheep and goats. It is caused by Mycobacterium avium subspecies paratuberculosis (MAP), an obligate intracellular pathogen that resides within macrophages and inhibits certain macrophage phagosomal and activation processes in order to survive (25, 50). MAP mainly resides in the gut mucosa and associated lymph nodes (LN) causing severe granulomatous enteritis and lymphadenitis, though dissemination in blood, milk and peripheral tissues occurs at certain times during infection (7, 15, 23). Exposure, generally thought to occur at a young age, is followed by a long (>1 year) subclinical or latent phase that in a proportion of animals progresses to clinical disease, characterised by wasting, diarrhoea and death (4, 13). The later stages of disease are associated with shedding of MAP organisms in the faeces, which contaminates the environment and results in infection of other animals (23, 29). Paratuberculosis has spread to every continent and disease control is limited by the difficulties associated with diagnosis of subclinically infected animals and lack of therapeutic options (51). Aside from animal welfare aspects, the disease has a major economic impact on the livestock industries due to stock and production losses (10, 23). Public health concerns have also arisen, with an association found between MAP and Crohn’s disease, a debilitating inflammatory bowel disease of humans (39, 44).
The mechanisms leading to the progression from subclinical to clinical disease are poorly understood in paratuberculosis as they are in other mycobacterial diseases. Immune regulation during chronic infection is required to eradicate the pathogen whilst minimising the destruction of host tissues. Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting enzyme in the breakdown of the essential amino-acid tryptophan within a highly conserved metabolic pathway (37). It was originally described for its antimicrobial role, depleting tryptophan essential for the growth of some microbial pathogens (53), but has also been found to be a potent immunoregulatory molecule in pregnancy and long-term immune responses including chronic infections and tumours (28, 37, 53). IDO is expressed by antigen (Ag) presenting cells such as macrophages (11) and dendritic cells (38) as well as neutrophils (8) and epithelial cells (19). The key cytokine that induces IDO expression is interferon-gamma (IFN-\(\gamma\)).

The immunoregulatory effects of IDO depend on T cells, which are very susceptible to tryptophan depletion and to its breakdown products (the kynurenines), leading to inhibition of T cell proliferation, anergy, increased T cell apoptosis, and also alteration of the Th1/Th2 balance (37). Tolerogenic DC expressing IDO have immunosuppressive functions and are involved in the generation of regulatory T cells (12, 38). Previous studies have indicated IDO may be induced in association with mycobacterial infections (1, 17), however nothing is known about the role of IDO in a natural host during the course of a chronic mycobacterial infection from exposure through latency to clinical disease.

The aim of this study was to examine immune regulatory pathways in MAP infection, particularly the tryptophan metabolic pathway and IDO, as a model for mycobacterial infection in a natural host. We hypothesised that IDO may be an important immune regulatory molecule in the progression of disease because macrophages are the target cell of chronic MAP infection and...
because the main site of infection is the gut, which is a highly regulated environment. In the first comprehensive study examining IDO gene expression, protein localization and functional analysis we show that high IDO expression at the site of infection and by peripheral blood cells is associated with clinical but not subclinical disease. IDO induction is associated with decreased plasma tryptophan levels; this precedes clinical signs and is not found in subclinical cases. These findings may have broader applicability to mycobacterial pathogenesis and inform studies of the processes involved in transition from latent to clinical disease.

MATERIALS AND METHODS

**Bacterial cultures.** MAP sheep strain Telford 9.2 (passage level 5) was reconstituted from lyophilised stock and inoculated into a radiometric BACTEC culture vial (Becton Dickinson) containing egg yolk and mycobactin J, cultured at 37°C for 2-3 weeks, then subcultured on modified Middlebrook 7H10 agar slopes supplemented with mycobactin J for 6 weeks, as previously described (4, 14). MAP cattle strain field isolate (CM00/416/C4) at passage level 6 (including its primary isolation from cattle faeces), was cultured as above. Total and viable MAP were enumerated by visual counts (Thoma-ruled counting chamber) using a standard three tube most probable number (MPN) method BACTEC culture (42).

**Experimental animals and tissue sampling.** All animal procedures were approved by the Animal Ethics Committee, University of Sydney. Merino sheep were sourced from properties in New South Wales that were either classified as free from JD (unexposed controls) or known to have endemic disease (naturally infected). JD-free farms were located in a region of Australia with negligible prevalence of JD, confirmed by negative serum antibody (Ab) ELISA and whole flock faecal culture. Disease status of naturally infected sheep was confirmed by serum Ab
ELISA, pooled faecal culture and/or specific IFN-γ assay, histopathology and mycobacterial tissue culture of culled sheep and direct faecal quantitative PCR (qPCR), as previously described (29, 48). Full details of individual animals, infection status and samples collected from naturally infected sheep have been previously published (48).

In addition, Merino lambs sourced from JD-free farms were used in two separate experimental infection trials (4). Lambs were sourced from the same farm and all tested negative for MAP infection using faecal culture, Ab ELISA and IFN-γ ELISA prior to the study. Trial A involved a comparison of three cohorts of sheep: 20 unexposed control sheep, 20 sheep inoculated at 3-4 months of age with MAP sheep strain (Telford 9.2) and 20 sheep inoculated at 3-4 months with gut homogenate from a clinically infected sheep (4, 14). The animals were managed under conventional Australian sheep farming conditions by grazing in paddocks with age-matched controls housed separately in paddocks where no MAP infected sheep had been kept previously. Trial B involved a similar design, comprising 20 lambs inoculated with MAP sheep strain (Telford 9.2) in three doses (4.3x10⁷, 2.3x10⁷ and 9.3x10⁷) with a total infectious dose of 1.6x10⁸ viable MAP, and 10 control unexposed sheep. An experimental infection trial in cattle (Trial C) was performed using a similar protocol based on the validated sheep infection model (4), but using MAP cattle strain. Twenty calves 3-6 months of age were inoculated with a low passage laboratory seed stock culture of MAP cattle strain (CM00/416/C4) in three doses over a one month period (2.3x10⁸, 9.3x10⁷ and 1.5x10⁸) with a total infectious dose of 4.8x10⁸ viable MAP (manuscript in preparation). Ten age-matched calves were used as controls.

Blood and faecal samples were collected from naturally infected animals and age-matched controls prior to necropsy. Animals in experimental infection trials were sampled prior to inoculation and then every 1-4 months. Blood samples were used for proliferation assays, RNA
isolation, whole-blood IFN-γ ELISA, and serum Ab ELISA (Porquier ELISA, IDEXX), as previously described (3-4, 14, 48) and plasma tryptophan analysis. Methods for peripheral blood cells isolation, whole blood IFN-γ ELISA and faecal culture for cattle were validated based on published methods used for sheep (manuscript in preparation). Necropsies were performed on all naturally infected sheep and sheep from experimental infection Trial A, as well as a subset of clinically diseased sheep from Trial B, as previously described (4). Briefly, multiple samples of intestinal tissue and the associated draining LN were collected, with tissues for RNA extraction and MAP culture immediately frozen and stored at -80°C. Tissues samples for histopathology and immunohistochemistry (IHC) were placed in 10% buffered formalin prior to being paraffin embedded.

Animals were classified by exposure history, histopathology results and tissue culture for MAP and divided into three groups: unexposed, uninfected (exposed but uninfected at time of necropsy) and infected (showing histopathological and tissue culture evidence of disease). Infected sheep were further categorised based on histopathological findings in the terminal ileum, according to Perez et. al.(1996), into low grade lesion (Grade 1-2), early paucibacilliary lesion (Grade 3a) or multibacilliary lesion (Grade 3b) cases (3, 41).

**Cell infection.** THP-1 cells, a human monocytic cell line, were sourced from the European collection of cell cultures and grown at 37°C and 5% CO₂ in culture media: RPMI 1640 media (Gibco) supplemented with 10% FCS (Invitrogen), 0.2mM L-glutamine, penicillin 100U/ml and streptomycin 100µg/ml (Gibco). Cells were plated 24hr prior to infection at 2x10⁵ cells/well in a 48 well plate tissue culture plate (BD Falcon) Cultures were either uninfected (control) or infected with MAP (Telford 9.2 sheep strain) at a multiplicity of infection of 10:1.
Four replicates for infected and 2-4 for control cultures were harvested at 0, 4, 8, 24 and 48 hr post-infection, resuspended in culture media, and centrifuged at 3000xg for 3 min prior to extraction of RNA for qPCR studies.

**Proliferation assay.** *In vitro* studies on proliferation of peripheral blood cells from sheep and cattle experimentally infected with MAP were performed using a fluorescent dye (CFSE) flow cytometric assay (14). Buffy coat cells were prepared by centrifugation of lithium heparin blood tubes at 1455xg for 20 min. The cells at the interface were aspirated and RBC lysed, then the remaining cells were washed with PBS and centrifuged at 233xg for 10 min and resuspended in culture media (as for THP-1 cell with 2-mercaptoethanol). These were cultured at 2.5x10^6 cells/well in triplicate in 96 well plates and stimulated with 10µg/ml MAP Ag (cattle strain 316v whole cell Ag, Elizabeth Macarthur Agricultural Institute, NSW, Australia), 5 µg/ml pokeweed mitogen (PWM) or culture media alone as a control. Replicate cultures were performed in the presence of the tryptophan analogue, 1-methyl-L-tryptophan (1-MT, Sigma-Aldrich), an inhibitor of IDO function. At the end of the culture period (6 days), samples were acquired on a flow cytometer (FACScan, BD), gated for lymphocytes and the percentage proliferation determined from the change in CFSE fluorescence.

**RNA extraction and cDNA synthesis.** Methods for peripheral blood mononuclear cell (PBMC) isolation from sheep blood, RNA extraction, DNase treatment of RNA and cDNA synthesis were previously described (48). For RNA extraction from cattle blood, buffy coat cells were prepared as above for proliferation assays from EDTA blood tubes and the cell pellet was extracted using the Illustra RNA spin mini kit (GE Healthcare). RNA was extracted from THP-1
cell cultures using the Illustra RNAspin mini kit. RNA quality was assessed using a spectrophotometer (Nanodrop, Thermo Scientific). 1-5µg RNA was DNase treated to remove genomic DNA, using 10U RQ1 DNase (Promega) and 1μl RNasin Plus RNase inhibitor (Promega), followed by synthesis of cDNA using AffinityScript reverse transcriptase (Agilent) according to the manufacturer’s instructions. cDNA was diluted 1/10 or 1/100 with nuclease-free water prior to qPCR analysis.

**Quantitative PCR (qPCR).** Primers were designed using Beacon Designer 4.0 (Premier Biosoft Int., Palo Alto, CA) or Primer3 software, with all sequences given in Table 1. As the ovine IDO sequence is not available, bovine/ovine specific IDO primers were designed based on the bovine IDO sequence (NM_001101866) and primers were selected that displayed strong homology (90%) between the bovine and equine IDO (XM_001490681.1), spanning a 396 bp intron (Table 1). Primers were optimized for use on both sheep and cattle cDNA. Reference genes for sheep tissues and PBMC were identified and validated in previous studies (48, 54). A reference gene for cattle qPCR analyses was identified from microarray studies as a stable, unregulated gene in peripheral blood cells at 9, 13 and 21 wk post-experimental infection (manuscript in preparation). For studies on THP-1 cells, human primers were designed and optimized for IDO, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-glucuronidase, Histone H3, interleukin (IL)-12B and IL-10 (Table 1). The reference gene (β-glucuronidase) for these studies was selected based on the method developed for MAP infection of RAW 264.7 cells (47).

qPCR was performed as previously described, using an Mx3000P Real-time PCR system (Stratagene, Agilent) and QuantiTect SYBR Green PCR kit (Qiagen) (48). Reactions (25µl)
contained 10ng template cDNA, or 5µl of a 1/10-1/100 dilution of cDNA, and an optimized
concentration of forward and reverse primers (300-600nM). qPCR experiments were run using
the following program: 95°C for 15min, then 40 cycles of 95°C for 20s, 52-60°C for 30s and
72°C for 30s, with fluorescence acquisition at the end of the annealing step. Reaction specificity
was confirmed post-amplification using a melt curve analysis. Standard curves were performed
for all primer sets once optimised using a dilution series (0.1-100ng) of cDNA (Table 1). Fold
change in gene expression was calculated using the 2^ΔΔCt comparative quantitation method (33),
following normalization against the appropriate reference gene. Data are presented as fold change
of gene expression using predicted means from the linear mixed model statistical analysis (see
below). This was calculated by comparison of the mean delta Ct for each treatment group relative
to the unexposed group, which was attributed an arbitrary expression level of 1. Significant
differences were also confirmed using Relative Expression Software Tool (REST 2008; Corbett
Research and M. Pfaffl).

Immunohistochemistry. IHC was performed on sections of paraffin embedded ileal
tissues from control and clinically infected sheep, necropsied 14-16 months after MAP
experimental infection. Sections were deparaffinised in xylene, then rehydrated through graded
ethanol baths. Ag retrieval was performed using Target retrieval solution, pH 6.1 (DAKO) and
boiling for 10min. Sections were washed with Tris buffer and endogenous peroxidase activity
was blocked using 3% hydrogen peroxide solution for 10min in a humidified chamber. Anti-
human/mouse IDO monoclonal Ab (clone 10.1, Millipore) that cross-reacts with bovine and
ovine IDO, was used at 2 µg/ml in Ab diluent (DAKO). Sections were stained with IDO Ab or
mouse IgG isotype control for 1 hour in a humidified chamber, washed with Tris buffer then
incubated for 1 hour with secondary Ab from the EnVision System-HRP kit (DAKO), followed by DAB substrate solution for 5min. Sections were counterstained with haematoxylin and bluing solution, dehydrated through ethanol and xylene baths and coversliped using DPX mounting media.

**Plasma tryptophan determination.** Blood was collected in lithium heparin vacuum tubes (Vacuette) from experimentally infected and control sheep and centrifuged at 1455xg for 20min. Plasma was collected and stored at -80°C. Tryptophan concentrations were determined using reverse-phase HPLC as described (34).

**Statistics.** Gene expression differences in monocylic cultures and within tissues and PBMC were examined using restricted maximum likelihood (REML) in a linear mixed model (GenStat release 12.1 2009, VSN International Ltd), as previously described (48, 54). For tissues, the analysis considered property of origin as a random factor, with fixed effects of exposure (unexposed, exposed), infection status (uninfected, infected) and histopathological lesion group (no lesion, early paucibacilliary, multibacilliary). Similarly, a REML linear mixed model analysis was performed on PBMC gene expression data that considered exposure, infection status and lesion group as fixed effects, with animal and time as the random terms to account for potential correlation across sampling timepoints and for individual animals. Significant differences in serum tryptophan levels were assessed using a Student’s t-test.

**RESULTS**
IDO is expressed by monocytes infected with MAP. To determine if IDO was induced in paratuberculosis infections, we first examined IDO gene expression by monocytes infected with MAP in vitro. IDO was expressed at low levels by uninfected THP-1 monocytes. Infection with MAP led to a rapid and highly significant (p<0.0001) induction of IDO gene expression (Figure 1A). This was evident within 4hr post-infection and reached over 75-fold of that of the control within 48hr. These results were confirmed in a second experiment, where IDO gene expression was induced >200-fold (data not shown). By comparison, IL-12 gene expression was induced about 6-fold and peaked at 8hr post-infection (Figure 1B).

To confirm the origin of the IDO expression, the MAP K10 complete genome (NC_002944) was searched but it did not contain a sequence similar to IDO, nor were the IDO primers used able to recognise sequences in the K10 genome, based on BLAST searches. Thus the increased expression of IDO was derived from the monocytic cells and not the intracellular MAP organisms.

Tryptophan biosynthesis pathway is present in MAP. Interestingly, the tryptophan biosynthesis (indole) pathway is present in MAP organisms, as shown in Figure 2. The K10 genome contains trpA (tryptophan synthase subunit alpha, MAP1307), trpB (tryptophan synthase subunit beta, MAP1306), trpC (indole-3-glycerol-phosphate synthase, MAP1305), hisA (phosphoribosyl isomerase A, MAP1297), trpD (anthranilate phosphoribosyltransferase, MAP1931c) and trpE (anthranilate synthase component I, MAP1303), which are involved in L-tryptophan biosynthesis. Thus MAP organisms may be protected from the effects of local tryptophan depletion due to IDO as they can synthesise this essential amino acid.
IDO is increased in the affected tissues of MAP infected animals. IDO gene expression was assessed in the tissues of the gut and draining LN, the sites known to be pathologically affected in animals with JD. Unexposed control sheep were compared to sheep exposed to MAP. Exposed sheep were classified at the time of necropsy as either uninfected or infected based on MAP tissue culture results and the infected animals were further categorised into low grade, early paucibacillary or multibacillary lesion types. Multibacillary lesions are the most severe, characterised by granulomas with high numbers of MAP organisms, and are generally found in the later stages of disease associated with clinical manifestations.

IDO mRNA was significantly increased in the gut tissue of MAP infected sheep compared to control unexposed animals (Figure 3A and B). Significant effects were identified using a linear mixed model that took into consideration the property of origin, with data (mean ± SEM) shown in Supplementary Figure 1, and also by REST analysis software (Supplementary Table 1). IDO expression in the ileum and jejunum of infected animals (combined paucibacillary and multibacillary cases) was significantly higher than unexposed controls and exposed uninfected sheep (p<0.005 and p<0.05 respectively). Multibacillary cases were associated with the highest IDO expression levels. The fold change in IDO expression was tended to be higher in the ileum (9-11 fold), the site in the gut where the disease is most expressed, though this did not reach significance. As animals were classified based on the most severe lesion type detected along the length of the gut (in the ileum), jejunum data could also be analysed based on the local lesion severity within these sections. When this was done, the IDO fold changes within this tissue were much higher (multibacillary 8.24 and paucibacillary 3.76 fold), indicating correlation of the IDO expression level with the local lesion severity.
Similar results were found in the draining LN, both ileal (Figure 3C) and mid-jejunal (data not shown), where infected sheep had significantly higher IDO gene expression than unexposed or exposed uninfected sheep (p<0.005 and p<0.05 respectively). As for the gut tissue, in LN the multibacilliary cases had the highest levels of IDO expression.

The increased IDO mRNA expression was confirmed to lead to increased IDO protein using immunohistology (Figure 4). There was strong staining of gut and LN tissues from sheep with granulomatous paratuberculosis lesions, both paucibacilliary and multibacilliary. IDO protein levels were low in the normal gut tissue of sheep, with light staining confined to crypt cells (Figure 4A). However in the infected ileum, IDO was strongly expressed by epithelial cells and also by macrophages, which were seen as ‘islands’ of staining in granulomas (Figure 4B and C). These same macrophage ‘islands’ in the multibacilliary lesions were the regions with the highest mycobacterial numbers, confirmed by Ziehl Neelson staining for acid fast organisms (Figure 4D). There was also strong staining for IDO by cells within the crypts of infected gut tissues that may be Paneth cells or macrophages entering the luminal space. Within the LN, IDO staining was associated with granulomatous foci comprised of infiltrating macrophages, typical of late stage disease (Figure 4F). These occurred within the parafollicular zones.

**IDO gene expression is increased in peripheral blood cells of MAP-exposed and infected animals.** IDO mRNA expression was measured in peripheral blood cells of sheep and cattle with JD (Figure 5, Supplementary Figure 2). When PBMC were examined from a group of naturally and experimentally infected sheep (1½-4 yr), late in the disease process, IDO expression was significantly increased (p<0.05) compared to unexposed controls (Figure 5A).
Further studies were conducted to examine the expression of IDO throughout the course of the disease.

Sheep were experimentally infected with a known dose of viable MAP organisms and blood was collected throughout the disease course for RNA extraction. The infection model has been validated for sheep and leads to similar disease progression and proportion of clinical cases as found with natural infections (4). Only a proportion of exposed animals subsequently develop clinical disease. Some animals that were exposed did not show signs of infection (termed ‘Uninfected’) as they were negative in tests to detect MAP shedding in the faeces and in tissue culture at necropsy. When unexposed, uninfected and infected cases were compared, sheep that were infected had significantly increased IDO expression (p<0.001) detectable in their peripheral blood late in the disease course (Figure 5B). Interestingly the exposed but uninfected animals had an early and significant spike in IDO expression (p<0.05) that later returned to baseline. The infected group was subdivided into low grade, paucibacilliary and multibacilliary cases, showing that the increased IDO gene expression was clearly associated with the multibacilliary cases (Figure 5C).

A similar experimental infection was conducted in cattle. Blood cells from exposed cattle (5 months post-inoculation) had significantly increased IDO expression (p<0.05) compared to unexposed controls (Figure 5D). The fold change in expression was highest in exposed cattle that responded early in a MAP-specific IFN-γ assay compared to those that did not respond (p<0.001).

Effect of IDO on responses of lymphocytes from experimentally infected animals.

Proliferative responses of peripheral blood lymphocytes (PBL) were examined to MAP Ag alone
and in the presence of the tryptophan analogue, 1-methyl-L-tryptophan (1-MT), an inhibitor of IDO. Studies were performed on both sheep and cattle comparing control unexposed and MAP experimentally exposed animals. An initial optimization of 1-MT concentration based on the reported effective dose range (34) was performed using blood cells from exposed cattle stimulated with MAP Ag or positive control (PWM) stimulation (Figure 6A). This showed that the best concentration was 0.5mM 1-MT, used in all subsequent studies.

Three clinically diseased sheep >12 months post-infection (experimental infection trial B) were analysed, all with acute weight loss, faecal shedding and severe granulomatous lesions associated with MAP, confirmed at the time of necropsy (Figure 6B). PBL from unexposed control sheep (n=3) proliferated to the positive control (PWM) but not specific MAP Ag, as expected from their exposure history (one representative animal shown). Of the three clinically infected sheep, two responded to MAP Ag in the proliferation assay (sheep B and C) while the third was unresponsive (sheep A). All responded appropriately to PWM, indicating that the cells were viable. The addition of 1-MT did not enhance the proliferative response of cells from control or diseased sheep to MAP Ag or PWM. Thus, blocking IDO by adding 1-MT did not restore MAP-specific proliferative responses of the non-responsive sheep.

Proliferative studies were also performed on four experimentally inoculated cattle at 17 months post-infection and three age-matched, unexposed control cattle (one representative control shown) (Figure 6C). Two of the cattle were shedding MAP in their faeces, detectable by faecal culture, but their PBL were unresponsive to MAP Ag (cow B and D). The other two were not shedding MAP and proliferated in response to MAP Ag. Similar to the findings in the sheep, the addition of 1-MT did not enhance the cellular proliferative response of any of the cattle, including the unresponsive infected cattle, to MAP Ag or PWM (Figure 6C). Thus, blocking the
effect of IDO \textit{in vitro} did not restore or enhance proliferative responses to MAP Ag of sheep with
JD or experimentally inoculated cattle.

\textbf{Decreased plasma tryptophan levels precede the onset of clinical disease.} Plasma was
collected at 14 months post-inoculation from control and exposed sheep from experimental
infection trial B. Within two months of this sampling, six of the sheep displayed clinical signs
consistent with JD including >15\% loss of body mass and shedding of MAP in the faeces,
detected by faecal culture. These sheep were sacrificed and necropsy confirmed clinical disease
associated with severe granulomatous lesions in the gut and dissemination of the infection to the
draining LN and liver.

The exposed sheep were categorised into those that developed clinical disease (at 14-16
months post-infection) and those that were exposed but did not show clinical signs. The clinically
diseased sheep had all shown a specific IFN-\(\gamma\) response during the course of the infection,
however two of these animals (both multibacillary cases) had a decline in their IFN-\(\gamma\) responses
concurrent with clinical disease onset. Of the exposed (subclinical) group, all except one had
shown a specific IFN-\(\gamma\) response over the course of the infection and 9/13 had evidence of
intermittent shedding of MAP, detected by faecal culture.

The plasma tryptophan levels are shown in Figure 7. The range of tryptophan levels in the
unexposed age-matched control sheep was 21.0 to 31.1 \(\mu\)mol/L. Sheep that subsequently
developed clinical disease showed a highly significant (\(p<0.00001\)) reduction in their plasma
tryptophan levels that preceded clinical signs, with a range of 9.1 to 18.8 \(\mu\)mol/L. This clinical
group also had significantly reduced plasma tryptophan levels compared to the remainder of the
exposed cohort that did not develop clinical signs (<0.0005). This indicated that tryptophan levels in the blood decreased, coincident with increased IDO expression in the gut and periphery.

**DISCUSSION**

This study examined the association of IDO with progression of paratuberculosis in sheep and cattle as a model for disease pathogenesis of virulent mycobacteria. We hypothesised that IFN-γ release at the site of immune activation may be associated with induction of IDO, a key immunoregulatory molecule. This could inhibit the local adaptive immune response to the infection, allowing the MAP organism to survive and contributing to the chronicity of the infection.

We found that IDO gene and protein expression was increased in infected tissues of clinically diseased sheep. Systemically, increased IDO gene expression could be detected in the peripheral blood cells of exposed sheep and cattle, particularly in the later stages and associated with severe disease. The IDO gene expression levels in the gut, the main site of infection, showed the highest fold change. This was particularly noted within the ileum, thought to be the primary site of infection (23) and reported to be the site of the gut more likely to have demonstrable evidence of disease and the most severe lesions (4, 15). IDO expression was also increased in other affected regions of the gut and associated LN, with the greatest change in expression found in animals with multibacillary lesions. These constitute a severe disease stage, with high numbers of acid fast bacilli and macrophages, reduced lymphocyte numbers and recently shown to represent an irreversible end-stage in the progression of the disease (16). Interestingly, increased IDO expression has also been found in intestinal lesions of human Crohn’s disease patients (2). In a study of cells in the sputum of patients diagnosed with active tuberculosis, IDO
was one of the genes that was significantly upregulated compared to healthy controls or patients with other lung conditions, suggesting a correlation with human tuberculosis (1).

Within the gut and LN of JD affected sheep, IDO protein was associated with granulomas, strongly suggesting expression by macrophages in these lesions. An interesting finding was the strong IDO staining of epithelial cells and possibly Paneth cells associated with JD pathology. IDO is expressed constitutively at low levels in the lower gastrointestinal tract, perhaps to limit the response to commensal bacteria (19). In inflammatory bowel conditions, IDO has been shown to be strongly expressed by epithelial cells, particularly those on the margins of ulcers or abscesses (19), consistent with our findings. Epithelial IDO expression is thought to play a dual role, acting as an anti-microbial barrier as well as limiting potentially damaging inflammatory responses. In JD, the finding of macrophage-associated staining, co-localised with high acid fast bacilli numbers, suggests that IDO expression does not limit the growth of MAP organisms. As will be discussed below, MAP may circumvent IDO by endogenous synthesis of tryptophan.

The evidence of systemic changes in IDO levels in peripheral blood cells associated with paratuberculosis is a novel and interesting finding. In sheep, modulated IDO expression was observed throughout the disease course, with greatest expression late in disease. Similar to findings in tissues, it was multibacilliary lesion cases that had highest peripheral expression of IDO. In early stages of disease in cattle, significant differences in IDO gene expression were also detected. The cell type producing IDO is likely CD14+ monocytic cells, as found in previous studies of human PBMC (11, 46). The profile of IDO gene expression in peripheral blood cells of uninfected animals, that were exposed but had no evidence of infection at the time of necropsy, was markedly different to the infected group, with an early peak in IDO expression that returned...
to baseline. It is interesting to hypothesise that these animals may have cleared the infection as
their IDO expression profile reflects an appropriate response, whereas the infected sheep did not
have this early response.

Despite evidence of IDO expression by peripheral blood cells, blocking IDO in vitro did
not enhance proliferation of lymphocytes from infected sheep or cattle. Some exposed animals in
this study did not show proliferation to MAP Ag, perhaps because it had not yet developed or the
response had waned. The unresponsive sheep was a multibacilliary case, a stage previously
reported to show generalized loss of immune responsiveness in IFN-γ and lymphocyte
proliferation assays (3, 14). Loss of responsiveness may be due to mechanisms such as anergy
and/or immunosuppression. Blocking with 1-MT may not have been effective for various
reasons; changes in the cell environment meant that the cells were no longer producing IDO,
available L-tryptophan levels were sufficient to overcome the effect of IDO-induced tryptophan
starvation, or the unresponsiveness was not due to IDO-induced suppression. Other forms of
immunosuppression may be involved, such as regulatory T cells. IDO may act indirectly via
induction of regulatory T cells (12), identified in chronic disease states including tuberculosis (5).
Alternatively, other cell types not present in the assay may produce IDO, as shown in mice where
IDO was expressed by non-hematopoetic cells (17). The effect of blocking IDO locally at the site
of infection by in vivo treatment with 1-MT cannot be assessed in large animals such as cattle and
sheep and reagents to further characterize the immune response in sheep and cattle are less
developed than in mice and humans. Studies in mice have shown that in vivo treatment with 1-
MT can reverse T cell inhibitory effects of IDO activation (43, 45).

Virulent mycobacteria such as Mycobacterium tuberculosis and MAP inhibit macrophage
processes to survive intracellularly, including phagosome maturation (25), apoptosis (27) and Ag
presentation (50). IFN-\(\gamma\), a potent activator of microbicidal functions of macrophages, is induced in mycobacterial infections and is essential for control of \textit{Mycobacterium tuberculosis} (21) as well as being a hallmark of MAP exposure (4, 26). Within tuberculosis lesions, IFN-\(\gamma\) is expressed by cells in the granuloma, yet despite this infection persists (18). This paradox has been partially explained by the finding that intracellular infection of macrophages with virulent mycobacteria inhibits macrophage responsiveness to exogenous IFN-\(\gamma\), with selective inhibition of genes normally induced by IFN-\(\gamma\) including MHC class II and CD64 (31).

The most potent known activator of IDO expression is IFN-\(\gamma\) and the IDO promoter has sequence elements that confer responsiveness to this cytokine (38). In subclinically affected cattle, the expression of IDO in peripheral blood cells correlated with MAP-specific IFN-\(\gamma\) responses. However in the later stages of disease, there was a lack of correlation between IDO levels and IFN-\(\gamma\) responses in the periphery, as the highest IDO expression was associated with multibacillary cases that have impaired IFN-\(\gamma\) responses (3). IDO was also highly expressed by monocytes infected with MAP. These were pure monocytic cultures with no source of IFN-\(\gamma\). Thus it appears that MAP infection itself leads to a rapid and marked expression of IDO.

Interestingly, IDO is one of the IFN-\(\gamma\)-inducible genes that is not inhibited by mycobacterial infection and for which expression was actually increased (31). We found IDO protein expression was associated with lesions that contained high numbers of MAP. Thus, the mechanism of induction of IDO in paratuberculosis may not involve only IFN-\(\gamma\) but also MAP infection of cells per se may induce high levels of expression. These findings posed the question; if MAP is known to affect numerous processes of macrophages to promote its survival, why is IDO spared and indeed selectively induced by this organism? Does it give MAP a survival advantage?
The increased expression of IDO associated with granulomatous lesions in JD may reflect an attempt by the host to control pathogen growth as well as limiting tissue damage caused by inflammatory immune processes. A study in gene knock-out mice, in which the non-hematopoetic cells were unable to respond to IFN-γ, suggested that modest expression of IDO may be important in preventing uncontrolled immune activation (17). Loss of IDO expression in this model was associated with an overwhelming immunopathology associated with neutrophils and Th17 activation and these mice succumbed to *Mycobacterium tuberculosis* infection.

However, in a mouse model of *Leishmania major* infection, IDO-mediated suppression of T cell responses in the draining LN was proposed to contribute to persistence of the pathogen (36). Similarly, we found high expression of IDO in multibacilliary lesions, which are characterized by reduced T cell numbers and increased mycobacterial replication (13).

Depletion of local tryptophan stores by IDO may not be detrimental to MAP survival. A number of pathogens have been reported to be susceptible to IDO mediated tryptophan starvation and restriction of growth (9, 24, 35, 49). This study suggests MAP is not one of these as it can synthesise its own L-tryptophan and IDO protein within tissues was found in regions of uncontrolled MAP proliferation. The tryptophan biosynthesis pathway is conserved in a range of pathogenic and environmental mycobacteria, including *M.tuberculosis*, *M.leprae* and *M.smegmatis* (KEGG pathway; Phenylalanine, tyrosine and tryptophan biosynthesis). Thus the implications for the survival of pathogenic mycobacteria of increasing IDO at the site of infection, based on lack of sensitivity to its effects, may be minimal. IDO is beneficial to the pathogen and not the host in this context, due to its immunosuppressive effects on host immune responses. This may represent the pathogen harnessing immune regulatory mechanisms of the host to obtain a survival advantage.
The granuloma has been considered to be a host defense mechanism that attempts to physically contain persistent pathogens by entrapment within an organised cellular ‘prison’. Alternatively, the granuloma can be considered as a stalemate between the host and the pathogen, such that there is control but not eradication of the infectious agent (6). Moving the balance towards excessive immune regulation could lead to local immunosuppression and favour the replication of mycobacteria, potentially breaking this stalemate. As the highest IDO expression is associated with later stages of disease, the associated local and systemic immunosuppression may contribute to the progression from subclinical to clinical disease. Immunosuppression and local enhanced proliferation of MAP may also facilitate dissemination, which is associated with severe disease in cattle and sheep (7, 15).

The fact that tryptophan levels in the plasma were reduced is indicative of a major systemic, functional effect of the increased IDO levels, rather than just an effect on local inflammatory processes. Most circulating tryptophan is bound to albumin in the blood, acting as a reservoir (40). The considerable reduction in total plasma tryptophan indicates prolonged IDO elevation which may be depleting body tryptophan reserves. A localised infection with more limited IDO elevation might be expected to affect localised tryptophan levels but not have such an impact upon total plasma tryptophan levels. The combination of chronicity, the large tracts of gut tissue that are pathologically affected and the peripheral expression of IDO appear to have led to a measurable effect on circulating tryptophan, which combined with malabsorption may contribute to the wasting seen in clinical JD. It may also contribute to the peripheral immune dysfunction in severe clinical disease. Serum levels of tryptophan are reduced upon activation of IDO in chronic immune conditions (52) and altered kynurenine:tryptophan ratios have been found in human inflammatory bowel disease patients (20). In pigs with induced lung inflammation,
518 decreased plasma tryptophan was proposed to relate to increased IDO activity in the tissues as
519 well as tryptophan usage to synthesise acute phase proteins (32). In a model of colitis in pigs,
520 administration of L-tryptophan led to improvement of clinical and histological signs, increased
521 daily weight gain and restoration of the integrity of the gut barrier (30). Dietary L-tryptophan
522 may be an option as a potential nutritional supplement in paratuberculosis, acting to reduce
523 immunosuppressive effects and weight loss associated with disease.

524 These studies identify a role for IDO in the pathogenesis of paratuberculosis. Tryptophan
525 depletion by IDO may not have the desired anti-microbial effect, as MAP can synthesise its own
526 tryptophan and is therefore not dependent on environmental sources. IDO and other
527 immunoregulatory mechanisms that are activated to limit immune mediated tissue damage may
528 be beneficial to the pathogen, enabling MAP persistence. The induction of IDO by MAP may be
529 another way in which the pathogen modulates macrophage responses to promote its survival.
530 Importantly, an increase in IDO expression and corresponding tryptophan depletion systemically
531 may signal a transition from subclinical to clinical disease, although it is not yet clear whether
532 this is a causal relationship. Studies conducted in model systems, such as experimental infections
533 using an IDO gene knockout host or mycobacterial deletion mutant strains unable to synthesis
534 tryptophan, may further elucidate this. These findings may have important implications for other
535 mycobacterial diseases, including tuberculosis in humans, giving insight into potential
536 mechanisms involved in the transition from latent to active disease.
ACKNOWLEDGEMENTS

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REFERENCES


CD8 T cells producing IL-12p40 mRNA in human lung tuberculous granulomas.

Immunology 105:325-335.


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47. Taylor, D. L., P. C. Thomson, K. de Silva, and R. J. Whittington. 2007. Validation of endogenous reference genes for expression profiling of RAW264.7 cells infected with...


**FIGURE LEGENDS**

**FIG. 1:** IDO gene expression is increased in MAP infected monocytes. Expression of IDO (A) and IL-12 (B) by the human monocytic cell line, THP-1, infected at a 10:1 multiplicity of infection with MAP. Gene expression was assessed by qPCR at early timepoints post-infection. Replicates for control uninfected (n=2 to 4) and MAP infected (n=4) cultures, harvested at 0, 4, 8, 24, 48hr post-infection. *p<0.05, **p<0.0001 compared to control cultures at the respective timepoint.

**FIG. 2:** Tryptophan biosynthesis pathway in MAP. Based on KEGG pathway mpa00400; Phenylalanine, tyrosine and tryptophan biosynthesis in *Mycobacterium avium* subsp. *paratuberculosis* (strain K10).

**FIG. 3:** IDO gene expression is increased in the affected tissues of JD infected sheep. qPCR results of IDO gene expression, normalized against reference gene, in the ileum, jejunum and LN draining the ileum. Fold change was compared to unexposed (control) group. Unexposed n=12; Uninfected (sheep exposed to MAP but uninfected at necropsy) n=6; Early paucibacillary n=6; Multibacillary n=6; Infected (combined early paucibacillary and multibacillary) n=12. Significant differences: a, p<0.005 compared to the same tissue in unexposed sheep; b, p<0.05 compared to the same tissue in exposed uninfected sheep.

**FIG. 4:** IDO protein is associated with granulomatous lesions containing acid fast bacilli. Immunohistochemical staining for IDO protein levels or acid fast bacilli within gut and LN tissues of sheep. A. Unexposed control sheep, ileum stained for IDO; B. Infected sheep ileum from a multibacillary JD case stained for IDO (brown); C. Infected sheep ileum highlighting region of diffuse IDO staining (brown) associated with granuloma (arrows); D. Same infected ileum section as C stained using a ZN technique to detect acid fast bacilli, visible as dark pink.
staining within the granuloma; E. Unexposed control sheep ileal LN stained for IDO; F. Infected sheep ileal LN stained for IDO, showing IDO staining (brown) associated with granulomatous lesions (arrows). Isotype control Ab staining was negative in all sections (not shown).

**FIG. 5:** IDO gene expression in peripheral blood cells is increased with MAP infection and modulated throughout the disease course. qPCR results of IDO gene expression, normalized against the appropriate reference gene, in peripheral blood cells from sheep and cattle. Fold change was compared to unexposed (control) group. A. Expression of IDO in PBMC from naturally and experimentally infected sheep at later stages of disease (1½ - 4 years of age). Unexposed (control) n=11; Uninfected (sheep exposed to MAP but uninfected at necropsy) n=5; Early paucibacillary n=7; Multibacillary n=7 and Infected (combined early pauci- and multibacillary) n=14. B and C. Timecourse of IDO gene expression in sheep from experimental infection Trial A. Unexposed (control) n=20; Uninfected n=10; Infected (combined early pauci- and multibacillary) n=18; Low grade lesion (infected with lesion grade 1-2) n=5; Early paucibacillary lesion n=8; Multibacillary lesion n=10. D. Expression of IDO in PBMC from experimentally infected cattle (n=20) and unexposed controls (n=10). Exposed cattle were further subdivided into those that responded in a MAP-specific IFN-γ assay (IFN-γ +ve; n=6) compared to those that did not respond (IFN-γ –ve; n=14), performed at 5 months post-exposure. Significant differences: a, p<0.05 compared to unexposed controls; b, p<0.001 compared to unexposed controls and uninfected group; c, p<0.01 compared to low grade and early paucibacillary lesion groups; d, p<0.001 compared to unexposed and IFN-γ –ve groups.

**FIG. 6:** Proliferation of PBL from MAP exposed and infected sheep and cattle. A. Titration of the concentration of IDO blocking agent, 1-MT (0 - 2mM) in CFSE proliferation assays of cattle peripheral blood cells. Cultures were stimulated with MAP Ag, PWM or media
control. Mean ± SD of percent proliferation, determined by decreased CFSE fluorescence compared to non-proliferating controls. Representative results shown of PBL from an experimentally exposed animal. B and C. Proliferation of PBL from sheep (B) or cattle (C). Cultures were stimulated with MAP Ag, PWM or media control with or without 1-MT. Mean ± SD of percent proliferation in CFSE assay. A representative unexposed control animal (sheep or cow respectively) was compared to individual infected animals. Media controls with or without 1-MT was not significantly different for all animals, therefore only the media/1-MT result is shown.

**FIG. 7:** Plasma tryptophan levels are decreased in clinically diseased sheep compared to control animals and MAP exposed subclinical cases. Results for individual animals with median of each group shown as a horizontal bar. Plasma was collected from experimentally exposed sheep at 15 months post-exposure. Clinically diseased sheep were identified in the 2 month period following sampling and necropsies performed to confirm disease status. Unexposed controls n=10, Exposed (subclinical) cases n=13, Clinically diseased cases n=6. Significant differences: a, p<0.00001 compared to unexposed control group; b, p<0.0005 compared to exposed (subclinical) group.
TABLE 1: Primer sequences and references.

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<thead>
<tr>
<th>Primer name</th>
<th>Gene</th>
<th>Sequence</th>
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<th>PCR Efficiency (%)</th>
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a. NCBI (National Centre for Biotechnology Information) nucleotide reference sequence number.
b. Efficiency of the primer set (%) in qPCR, determined from cDNA standard curve.
c. Bov RG: reference gene for cattle PBMC identified by microarray studies, predicted bos taurus.
d. zinc finger CCCH-type containing 13 (ZC3H13), Affymetrix ID Bt.28167.1.S1.
d. Human β-Glucuronidase was selected as the reference gene for THP-1 studies as it had the lowest SD (0.18) across treatment groups (control and MAP infected) and timepoints (0, 4, 24 and 48hr) at levels closest to the genes of interest (48).
A

**IDO**

- MAP infected
- Control

B

**IL-12**

- MAP infected
- Control