The Paratuberculosis Newsletter

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CONTENTS

Note from the Editor

In this edition Dr William Davis presents a comprehensive informative report on methods to study immune responses in MAP infections.

Kumi de Silva

IAP business

None to report for this quarter.

Paratuberculosis News

Follow news about the next ICP in Dublin on twitter @para_tb2020

Canadian Cattlemen magazine recently reported on research by Dr Lucy Mutharia, University of Guelph which identified MAP antigens with potential for improving diagnostic tests.

The Western Producer interviewed Dr Herman Barkema, University of Calgary who studied farms participating in ProAction (a national program developed by Dairy Farmers of Canada) and found that Johne's prevalence could be decreased in part through management changes to calving pens, calf liquid diets and separation of cows and young stock.

Protocols to study the immune response to *M.* subsp. *avium paratuberculosis* ex vivo

William C. Davis Washington State University Pullman WA

When we first attempted to study the immune response to *M a paratuberculosis* (*Map*) with Rod Chiodini in the late 1980s, most of the information available on the immune response to *Map* was derived from studies in mice. Monoclonal antibody (mAb) reagents were just becoming available for research in cattle. A question of the time was whether exposure to *Map* always led to infection. There was also a question of whether there was a difference in susceptibility to infection associated with age, with young animals apparently more susceptible than older animals to infection. Tracking the cellular response to *Map*, purified protein derivative (PPD), or preparations of soluble antigens ex vivo was measured by use of tritiated thymidine incorporation (2). Multiple advances that occurred over the ensuing years increased our understanding of the immune response to *Map* and led to the development of methods to use cattle to study the entire cellular immune response to *Map* ex vivo (1). Because of the importance of this advance, I am taking this opportunity to describe the methodology to the members of the IAP with a brief history of the studies leading to the development of the methods to study the immune response ex vivo. The development of mAbs to bovine leukocyte differentiation molecules and the use of flow cytometry improved our ability to characterize the cells proliferating in response to antigenic stimulation and demonstrate all animals, exposed to *Map* under experimental conditions, become infected and develop humoral and cellular immune responses (3). Studies with newborn calves revealed a cellular response could be detected in all exposed calves within the first months following infection (3, 4). Development of a method to cannulate the ileum in 4 – 6 month old calves allowed for the demonstration that *Map* is rapidly taken up and disseminated to other tissues without establishing any lesions in the ileum during the first eighteen months post exposure (5). Improvements in ex vivo methods of culture revealed a proliferative response to *Map* antigens could be consistently detected four months post exposure (6). A characteristic of the response was that regardless of the type of antigenic stimulus, it always elicited a response that involved both CD4 and CD8 T cells in animals experimentally and naturally infected with *Map*. Further opportunity to analyze the importance of the dual response to antigenic stimulation occurred following the sequencing of the *Map* genome and examination of the immune response to expressed *Map* proteins and mutants of *Map* with random and targeted deletion of genes associated with virulence (7-11). Of particular interest, deletion of *relA*, a global regulator of multiple genes, disrupted the capacity of *Map* to establish a persistent infection. Comparison of the immune response to the mutant with the response to the wild form of *Map* revealed no apparent differences in the proliferative response (10). However, examination of calves exposed to the mutant or wild type *Map* and then challenged with wild type *Map* demonstrated there was a clear difference in the immune response. Examination of all tissues from the calves exposed to the *relA* mutant, by culture for bacteria and PCR, revealed infection with the mutant was cleared. Examination of tissues from control calves exposed to Wild type *Map* showed *Map* was not cleared. Comparison of the bacterial load in calves exposed and challenged with wild type *Map,* with calves exposed to the mutant and challenged with *Map,* revealed the bacterial load was greatly reduced in the mutant-exposed calves. This observation suggested the inability of the *relA* mutant to establish a

persistent infection was attributable to development of an immune response that cleared infection with the mutant (12). Development of a mAb to CD209, a molecule exclusively expressed on conventional myeloid dendritic cells in blood (cDC) and monocyte derived dendritic cells (MoDC) provided an opportunity to explore this possibility (13). Monocytes could be isolated from PBMC to generate MoDC or monocyte derived macrophages (MoMΦ) for use as target cells. Monocyte depleted PBMC (mdPBMC) containing cDC could be used for primary antigenic stimulation of CD4 and CD8 T cells or antigenic stimulation with MoDC pulsed with antigens. Individual animals could be used as a continuous source of cells, avoiding any issues associated with differences in genetic background. The assay protocol for analysis of the recall response in vaccinated animals is illustrated in Fig. 1. Use of the assay with a steer vaccinated with the *relA* mutant revealed a strong recall response could be elicited with stimulation of monocyte depleted PBMC (mdPBMC) with preparations of the live *relA* mutant. An identical response could be elicited with MoDC pulsed with the mutant. The response included a strong proliferative response by both CD4 and CD8 T cells (13). Of special interest, comparative studies revealed an identical recall response could be elicited with MoDC pulsed with a major membrane protein (MMP) encoded by *Map2121c*.

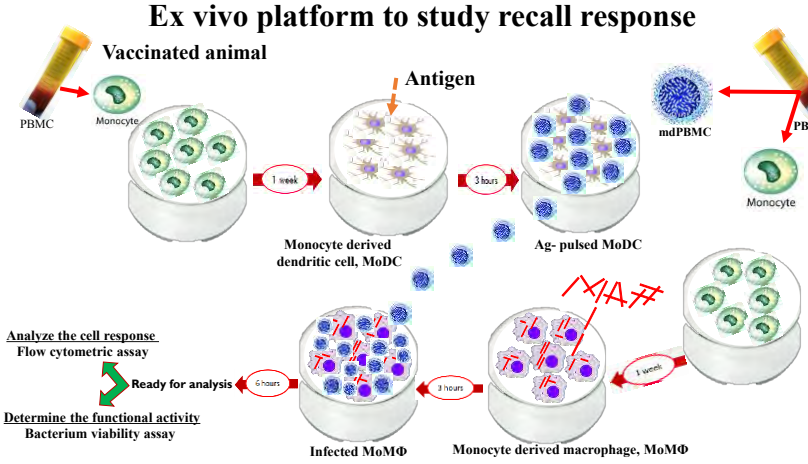

Fig. 1. Details of the assay are described in (1)

Further development of the ex vivo assay required improvement in methods to distinguish live from dead bacteria in a mixed population of live and dead bacteria and methods to determine the functional activity of the CD4 and CD8 proliferating in response to stimulation with MoDC pulsed with the *relA* mutant and MMP. Concurrent studies by Kralik et al. revealed a fluorescent dye, Propidium monoazide (PMA) could be used in an assay with a single gene probe (F57) and quantitative PCR, to distinguish live from dead bacteria (14). Exploratory studies revealed the assay could be used to distinguish live from dead bacteria present in experimentally infected MoMΦ. The relative percent of live and dead bacteria could be determined using a standard DNA curve determined with known concentrations of single copies of *Map* DNA. The assay proved to be more accurate than the CFU assay and provided a more expedient, direct assessment of killing of *Map* in infected target cells by cytotoxic T cells (CTL) (Fig. 2). A method developed by Worku and Hoft was adapted for determining the functional activity of CD4 and CD8 T cells (15). In their assay, MoMΦ target cells infected with *Mycobacterium bovis* BCG were overlaid with BCG or antigen stimulated lymphocytes from humans with latent infection with *Mycobacterium tuberculosis Mtb* or vaccinated with BCG. Following co-cultivation, cell preparations were lysed to free BCG. Inhibition of growth (inferred intracellular killing) of bacteria was determined by incorporation of tritiated uridine (16). In the modified assay (Fig. 2), live wild type *Map* were used to infect MoMΦ target cells.

The viability assay was replaced with the PMA-based viability assay. Infected target cells were overlaid with cultures of mdPBMC from a vaccinated steer stimulated with the *relA* mutant or MMP. The assay revealed stimulation of mdPBMC from a vaccinated steer with MoDC pulsed with either the mutant or MMP elicited the proliferation of cytotoxic T cells (CTL) with the ability to kill intracellular bacteria (Fig. 2).

The last step in development of the ex vivo assay revealed it is possible to study the entire immune response to *Map* and MMP entirely ex vivo. As illustrated in Figure 3, two rounds of stimulation of mdPBMC from unvaccinated steers, first with cDC (present in the mdPBMC) and then with MoDC pulsed with the *relA* mutant or MMP elicited the development of CTL with the ability to kill intracellular bacteria (1).

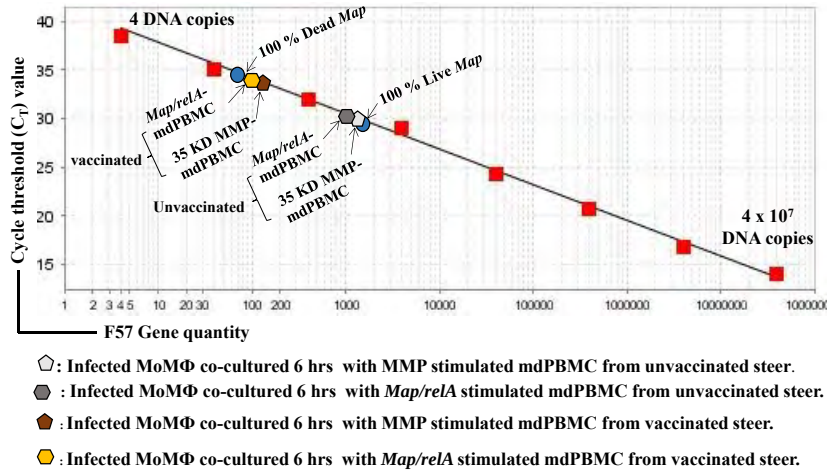

Fig. 2. Details of the assay are described in (1)

To date, our use of the assay has provided data showing the CTL activity present in stimulated mdPBMC is mediated primarily by CD8 T cells with a suggestion that some killing activity may be mediated by CD4 T cells. No killing activity was detected with NK cells and γδ T cells present in the cultures (1). Of special interest the most recent finding is that development of CTL activity requires the simultaneous recognition of antigenic peptides presented to CD4 and CD8 T cells by antigen pulsed DC. Analysis of the mechanisms of killing show the perforin granzyme B pathway is used to kill intracellular bacteria. Granulysin may be involved in delivery of the fatal hit by granzymes B (1).

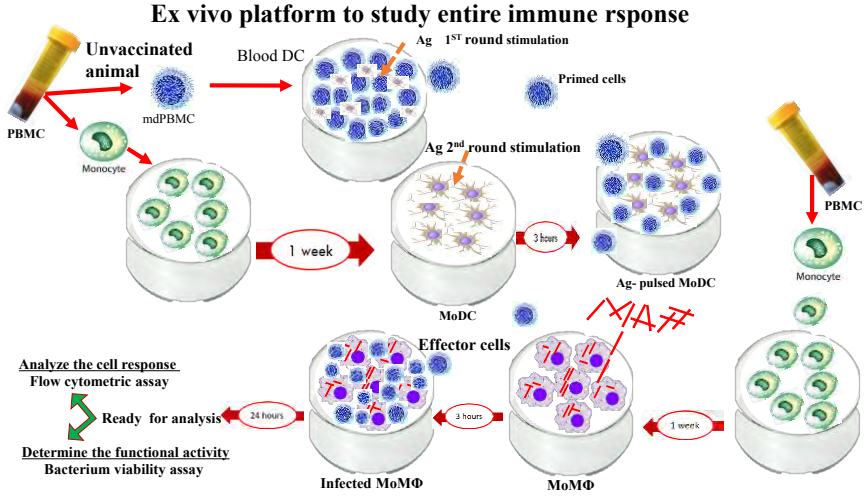

Fig. 2. Details of the assay are described in (1)

It is hoped that use of the newsletter introduces these advances in methodology to all investigators conducting research on the immune response to *Map*. We have only used the assays in cattle, but the assays have universal potential. They can be used with PBMC from other ruminants as well as with PBMC from humans, allowing for direct comparison of potential efficacy of candidate vaccines.

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Upcoming events

- International Veterinary Immunology Symposium 2019 in Seattle, USA

- 15th ICP on 13-18 June 2020 in Dublin, Ireland

- 7th International Conference on *Mycobacterium bovis*

- 16th ICP in 2022 Jaipur, India

Have you attended a conference recently where there were presentations related to paratuberculosis? Email editor@paratuberculosis.net to share this information

Recent publications (December 2018-March 2019)

Aboagye, G. and M. T. Rowe (2018). Evaluation of denaturing gradient gel electrophoresis for the detection of mycobacterial species and their potential association with waterborne pathogens. J Water Health **16**(6): 938-946.

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Rehman, A. U., M. T. Javed, M. S. Aslam, M. N. Khan, S. M. Hussain, K. Ashfaq and A. Rafique (2018). Prevalence of paratuberculosis in water buffaloes on public livestock farms of Punjab, Pakistan. Vet Ital **54**(4): 852.

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Deadline for next issue: 15 May 2019

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