Evaluating the *in vivo* immune response to *Mycobacterium avium subspecies paratuberculosis* infection in naïve and vaccinated calves

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

To my parents, siblings, husband, and M, C, H. Thank you.

TABLE OF CONTENTS

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ABSTRACT

In these studies, an implant device was developed to detect vaccination-induced, cell-mediated immunity in the subcutaneous tissue of calves. The long-term, future aim of these combined studies is to use the implant device to efficiently screen novel *Mycobacterium avium* subsp. *paratuberculosis* (MAP) vaccine candidates. This *in vivo* screening test would rapidly and inexpensively provide knowledge of a vaccine's efficacy in the host species; vaccine candidates exhibiting evidence of efficacy could then be selected for more extensive vaccine challenge studies. A better understanding of *in vivo* cell-mediated immunity in response to MAP immunization is essential for the development of a novel vaccine screening test. In all of the studies presented, Mycopar® vaccinated bull calves were compared to unvaccinated, control calves. Our first specific aim was based on a proof-of-concept study that focused on the development of a platform for *in vivo* detection of cell-mediated immunity in cattle. The results of the study showed that MAP-specific *in vivo* measurements can be achieved with the use of our designed implant device. Histological analysis revealed a lack of cellular infiltrate in naïve calves as well as the vaccinated calf not challenged with the MAP antigen, purified protein derivative-johnin (PPD-J). This demonstrated antigen-dependent immune cell recruitment into the collagen of the implant and illustrated the collagen's capacity for cellular migration. IFNγ was only detected in the antigen-containing implants that were placed in vaccinated calves. IL-10 did not exhibit antigen or vaccine-dependent trends. In a longitudinal analysis, the implants were measured at 0, 2, 4, 6, and 8 weeks post-vaccination. IFNγ levels in the MAP antigen-containing implants placed in vaccinated calves were significantly higher than the implants retrieved from naïve cattle at weeks 4

and 6 post-vaccination. These responses paralleled the responses observed in MAP-stimulated peripheral blood leukocytes (PBL) from vaccinated calves. Significant differences in IL-10 levels between the implants within vaccinated and naïve calves were not observed. The intradermal caudal fold test (CFT) demonstrated false-positive rates when one of our calves reacted to PPD-J prior to vaccination. This indicated that our implant device was capable of identifying each of the 3 vaccinated calves, suggesting an increased specificity over the intradermal CFT using PPD-J. Using live MAP bacteria, we explored the device's potential as a bacterial killing assay; collagen and bacteria-containing implant devices were placed in the subcutaneous tissues of calves divided into two groups, naïve and vaccinated calves, at 14 and 20 days post-vaccination. After 5 days, implants were retrieved from the subcutaneous tissues and collagen was processed for evaluation of IFNγ cytokine levels and flow cytometric analysis of bacterial viability. Three weeks post-vaccination, bacteria was removed from the 5-day implanted collagen, and flow cytometric analysis of the bacteria demonstrated a significantly greater percentage of propidium iodide (PI) stained-MAP in the vaccinated calves compared to the naïve calves. This finding indicated an enhancement of bacterial killing within collagen implants placed in vaccinated calves. Collagen from vaccinated calves at the three week post-vaccination time point also demonstrated a significantly higher production of IFNγ than collagen from naïve calves. The work of this dissertation provides supporting evidence that a subcutaneous bacterial challenge model could prove to be an efficient and cost-effective method for screening novel vaccine candidates in a natural host model.

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

Dissertation Organization

This dissertation is organized in the journal format in which each chapter is an independent manuscript. The chapters follow the submission guidelines for the specific journals in which they will be submitted. Chapter 1 is comprised of an overview and literature review and is followed by three individual manuscripts (Chapters 2-4). Chapter 5 contains general conclusions and future directions with references cited at the end of each individual chapter.

Disease Overview

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis in ruminants around the world. MAP infection, also known as Johne's disease, is characterized by chronic granulomatous enteritis resulting in a general wasting condition noted by the clinical signs of diarrhea, weight loss, and loss of milk production [1-3]. The greatest risk of infection occurs during the neonatal period, and vertical transmission from cow to calf is considered the most common route of infection; however, horizontal transmission from calf to calf or newly acquired cow is also observed [1, 4]. It is well established that calves are most susceptible to MAP infection the first few days to months of life [1, 4, 5]. Age-related MAP resistance has been demonstrated and by one year of age, cattle are considered low risk for infection [4]. The majority of calves that are exposed to MAP do not develop chronic wasting disease. In fact, only 10-15% of exposed calves will progress with symptoms of clinical disease after a prolonged incubation period that is known to last from 2-10 years [1, 4]. During this phase of infection, known as Stage I, clinical signs

and bacterial shedding do not occur; therefore, detection of MAP in this stage of disease is particularly difficult. Stage I animals contribute to the spread of infection in a herd by progressing to the advanced disease stages (Stages II-IV) that are known to shed MAP into the environment and eventually contribute to significant economic losses [1]. The annual monetary loss due to low milk production and early cull rates is estimated between $200-500 million with at least 68% of dairy herds in the United States containing MAP infected cattle [1, 6]. Treatment is not typically an option due to the high cost of antimycobacterial therapeutic agents combined with the high doses required for long treatment periods. Current methods of control include reducing MAP infection within a herd by testing and removing infected cows, prevention of new infections, vaccination, and genetic selection [1, 7]. Unfortunately, current diagnostics are infrequently able to detect subclinical stages of infection (Stage I, II). Due to difficulty in identifying MAP-infected animals, vaccination is increasingly looked to for control and elimination of MAP from cattle herds. The currently available vaccines do not fully prevent infection and may interfere with diagnostic testing for *Mycobacterium bovis*, although they do decrease fecal shedding [1, 7]. Development of new vaccines against paratuberculosis is an area of active research; however, an obstacle hindering the development of new, more effective vaccines is the difficulty in testing vaccine candidates. This is due to the long incubation period following infection that causes bovine vaccine challenge studies to be time-consuming and expensive. Furthermore, alternative models using rodents and cell culture systems do not accurately predict the bovine immune response to vaccines [3, 8]. The projects included in this dissertation are directed toward better understanding the early, post-vaccination, cell-mediated immune response in cattle to facilitate the development of an improved model for challenging MAP vaccine candidates.

Specific Project Aims

The objective of the research contained in this dissertation was directed at achieving a greater understanding of the early, *in vivo*, cell-mediated immune response in cattle vaccinated against MAP. Detection of cell-mediated immune responses is a central component of testing new vaccines against mycobacterial infections, and the long-term goal of these studies is to develop a novel screening tool to evaluate the efficacy of MAP vaccine candidates. This *in vivo* screening test would provide knowledge of vaccine efficacy for a target species in an inexpensive and short time frame. Vaccine candidates that show evidence of efficacy could be moved to more thorough vaccine challenge studies in calves. To achieve this goal, we first developed a novel subcutaneous implant device to evaluate the subcutaneous immune responses occurring post-vaccination (Chapter 2). Our hypothesis for these studies was that the implant device will detect vaccination-induced cell-mediated immunity in vaccinated calves as well as serve as a mechanism to measure MAP viability post-vaccination. To test our hypothesis, our first study was a longitudinal investigation evaluating the device's success at identifying vaccinated calves from naïve calves (Chapter 3). Our final aim was to use the device to implant live MAP subcutaneously and detect immune responses to live bacteria and assess bacterial viability (Chapter 4).

Literature Review

Adaptive immunology of MAP infection: Introduction

It is well understood that the adaptive immune system plays an important role in the early course of MAP infection in cattle. It has been shown that the early stage of infection begins with a cell-mediated immune response that later switches to an ineffective humoral response as the disease progresses past the subclinical phase [9-12]. This cell-mediated immune

response begins when orally ingested bacteria travel to the ileum where they are phagocytized by the microfold (M) cells within the Peyer's patches and transported to the submucosa. Here, bacteria are ingested by epithelial macrophages and dendritic cells [4, 13, 14]. Following the uptake of bacteria in the ileal macrophages, an appropriate T helper 1 (Th1) immune response ensues and is primarily directed by type 1 CD4+ T cells [9, 10]. An effective Th1 response is necessary for controlling disease and is characterized by the release of pro-inflammatory cytokines, such as IFNγ [12, 15]. The transition from the subclinical to clinical stage of disease occurs with deterioration of the Th1 response and development of a T helper 2 (Th2) immune response and is characterized by production of Th2 cytokines, such as IL-10 [16]. Secretion of Th2 cytokines facilitates a humoral immune response by stimulating B lymphocyte proliferation while inhibiting Th1 cytokines [17-19]. This review will highlight the Th1/Th2 paradigm, delayed-type hypersensitivity (DTH) intradermal skin test, and cytokines associated with the adaptive immune response observed in MAP infection.

Th1 to Th2 paradigm

The host immune response to MAP infection is complex, with cell-mediated immune responses seen during the early, subclinical stage of infection, and humoral responses observed during the late, clinical stage of infection. Investigations in cattle and mice have demonstrated the ability of IFNγ and the Th1 response to contain infection and halt disease progression [16, 20]. Other studies have shown early stage lesions to have high levels of IFNγ associated with a Th1 immune response [16]. It is plausible that the Th1/IFNγ response is contributing to the protective response typically observed in the subclinical stage; evidence

to support this includes IFNγ's ability to initiate killing of MAP within macrophages [21]. Begg et al. used naturally infected sheep to show a correlation between lesion severity and Th1/Th2 response. In the study, MAP-infected sheep with less extensive lesions had stronger Th1-type cytokines present [9]. A second sheep investigation looking at antigen-specific IFNγ levels demonstrated an association between low IFNγ levels and an increase in the severity of ovine paratuberculosis [22]. *In vitro* bovine monocyte studies have shown a decrease in IFNγ signaling associated with MAP-infected monocytes [4, 22]. Numerous murine models have been used to study the Th1 response in mycobacterial infections. Orme et al. demonstrated that mice injected with *M. tuberculosis* exhibited a high level of IFNγ for 30 days post-infection. After thirty days post-infection of mice, IFNγ levels dropped and an increase in IL-4 levels were observed indicating the switch from a Th1 to a Th2 response, respectively [23]. A second murine study used IFNγ knock-out mice to demonstrate the importance of IFNγ signaling and the Th1 response during early infection. This study showed disseminated tuberculosis in mice with a knock-out of the IFNγ gene [24]. These studies described in cattle and mice have demonstrated that MAP infection induces a Th1 protective response capable of containing infection and halting disease progression.

The transition from the subclinical to clinical disease state is associated with the differentiation of naïve CD4+ T-cells to a Th2 immune response. The production of Th2 cytokines support a humoral immune response by stimulating the proliferation of B lymphocytes and inhibiting Th1 cytokines [25]. Various studies in ruminants and humans have validated the correlation between mycobacterial disease progression and a Th2 immune response. As previously stated, Begg et al. was able to show that MAP-infected sheep with a predominance of Th1-type cytokines had less extensive intestinal lesions. In contrast, sheep

with late stage disease and more extensive intestinal lesions had higher levels of MAP-specific IgG antibodies indicating the predominance of a Th2 immune response [9]. A different study in humans showed the Th2 response and its association with disease progression. Seah et al. measured IL-4 mRNA levels in *M. tuberculosis*–infected humans; increased levels of IL-4 mRNA were observed with patients in advanced diseased states [26]. The results of these sheep and human studies support a switch from a Th1 to a Th2 immune response that is correlated with progression of mycobacterial disease.

Presently, the cause of the Th1/Th2 shift is still unknown. However, numerous theories for the Th1/Th2 immune paradigm switch have been proposed and include periparturient hormonal fluctuations, bacterial load, T cell exhaustion, and regulatory T cells (Treg) limiting Th1-type responses. In some recent long-term studies using experimentally infected sheep models, the traditional Th1 to Th2 transition has not been observed. In fact, these studies show that early in the course of infection both Th1 and Th2 responses are present [27, 28]. For instance, Waters et al. used experimentally infected calves to longitudinally evaluate IFNγ levels and humoral antibody levels by absorbed ELISA. This study showed MAP-specific antibody detection in the early stage of experimental MAP infection before clinical disease is observed [28]. A separate investigation in sheep by Begg et al. concluded that the Th1/Th2 immune responses in an ovine model are more complex than previous investigations indicated. Begg et al. used sheep to demonstrate both antibody and cell-mediated immunity as key players in early MAP infection. The results of this study showed 50% of sheep have a combined IFNγ and antibody response and 39% of sheep demonstrated a switch from IFNγ to predominately antibody production. The sheep with more extensive disease were found to have a reduced Th1 response characterized by a decrease in functional

ability to produce IFNγ [9]. Mathematical modeling of MHC Class II-mediated immune responses has been demonstrated in tissues [85]. In a new study by Magombedze et al., the previous data by Begg et al. was reexamined via a mathematical model. In this study, 39% of sheep exhibited a classical Th1 to Th2 switch, 50% exhibited a combined Th1 and Th2 response, and 11% exhibited only a Th1 response [29]. There is still ambiguity in the literature regarding the differences in Th1/Th2 responses among infected sheep. This novel mathematical model is used to evaluate the dynamics of MAP-specific Th1 and Th2 responses. The mathematical model was based on known properties of mycobacterial infections, experimental knowledge, and a theoretical understanding of Th1 and Th2 cells compared to naïve T cells (Th0). The results of this study suggested that when a Th1 to Th2 transition occurs it may be initiated by the accumulation of long-living extracellular bacteria, which contradicts previous theories in which the Th1 to Th2 switch was believed to come first and induce a proliferation of the bacterial population [29]. This mathematical model, along with the recent longitudinal sheep studies described, has shifted the discussion of cell-mediated immunity and the Th1/Th2 paradigm in mycobacterial disease toward a potential protective role of the humoral response in early infection.

Delayed-type hypersensitivity and johnin intradermal testing

The cell-mediated immune (CMI) response in cattle is considered the initial host response against mycobacterial infections [9, 29]. Therefore, CMI testing may be a useful tool for early detection of infection and as a screening test for vaccine efficacy trials. Currently, evaluation for the presence of CMI in humans and animals often includes delayed-type hypersensitivity (DTH) intradermal testing. French physician Charles Mantoux originally developed the currently used Mantoux tuberculin skin test (TST) in 1912 from the work of

previous scientists [30]. The DTH response, also known as a Type IV hypersensitivity response, appears hours to days after antigen exposure and represents an immune reaction mediated by T lymphocytes. The pathogenesis of the DTH intradermal skin test begins with the phagocytosis and processing of antigen by antigen-presenting cells (APC) such as dendritic cells and macrophages. The inflammatory reaction that ensues is caused by Th1 cells that infiltrate the site of antigen exposure and recognize complexes of peptide: MHC class II molecules on APCs. This peptide recognition between a previously primed Th1 cell and an APC is followed by secretion of pro-inflammatory cytokines such as IFNγ. The pro-inflammatory cytokines then act on local vascular endothelium to stimulate vessel permeability allowing plasma and an inflammatory cell infiltrate to enter the antigen exposure site. The mediators released by Th1 cells in the DTH response include chemokines and cytokines that facilitate recruitment of macrophages to the site. IFNγ is one of the most well-known cytokines in the DTH response; IFNγ activates macrophages and induces vascular adhesion molecule expression [31]. IFNγ will be discussed in more depth in section 1.3.

The Johnin skin test for detection of MAP infection is a well-known CMI test modeled after the Mantoux tuberculin DTH test described above. Similar to the tuberculin skin test, ruminants are injected intradermally with 0.1 ml of 1-1.5 mg/ml purified protein derivative-johnin (PPD-J) and skin thickness is measured 72 hours post-injection [32-34]. Two advantages of the intradermal DTH test compared to other CMI testing methods, such as the IFNγ assay, include the ease of performance and the low costs associated with performing the test. Studies have shown the Johnin DTH test to have >98% specificity in sheep and >93% specificity in dairy cattle [32, 33]. These investigations demonstrate good specificity in

MAP-free herds and flocks; unfortunately, this level of specificity has not been demonstrated in MAP-infected herds [34]. Investigations by Robbe-Austerman et al. studying sheep found the skin test was specific (98.7%) in non-infected flocks and sensitive (73.3%) in infected flocks; the sensitivity of the skin test exceeded that of serologic tests in infected flocks. The cattle and sheep in infected herds and flocks have an increased positive skin test response but do not show infection grossly or histologically on necropsy examination [32, 33]. A proposed explanation for the poor sensitivity found in MAP-infected herds is the poor sensitivity of reference tests to identify every infected animal. It has also been theorized that uninfected animals with previous exposure respond to the johnin antigen; it has also been proposed that previously infected animals have recovered prior to necropsy evaluation. One investigation compared the CMI response in sheep by exposing sheep to antigen by either inhalation or oral ingestion of dead organisms. The findings suggested that a positive skin test result is not observed with exposure to dead organisms via inhalation or ingestion indicating that sheep are either no longer infected at necropsy or reference tests lack sensitivity [34]. Variability in specificity is not only observed between infected and non-infected populations but variation has also been shown in different batches of PPD-Johnin [32]. The influence of antigen batch on specificity has been previously demonstrated in studies investigating bovine tuberculosis using the *M. bovis*-PPD [35, 36].

The studies discussed above investigating johnin DTH skin test specificity did not include evaluation of vaccinated animals. Hines et al. evaluated the specificity of the johnin DTH skin test in MAP-vaccinated goats. The study used a caprine model to look at various immune parameters following vaccination with 5 experimental, attenuated strains of MAP vaccine candidates compared to the commercial control vaccine, Silirum®. Intradermal skin

testing was performed at various time points before and after vaccination using three types of PPD: PPD-*M. avium*, PPD-*M.bovis*, and PPD-johnin. False-positive PPD skin test reactions were commonly observed in all groups for *M. avium*, including prior to vaccination. Post-vaccination, false-positive skin test reactions for each of the PPD groups were observed as such: false-positives for PPD- *M. avium* in the control group, Silirum® vaccine, 3 of the experimental vaccine groups, false-positives for PPD-johnin in the Silirum® vaccine and 1 experimental groups, and false-positives for PPD-*M. bovis* in the Silirum® vaccine and 2 experimental groups. The results from this study showed that the commercial vaccine, Silirum®, as well as some of the experimental vaccines, produce false-positive results with all three PPD skin tests [37]. These findings are particularly significant when considering the utilization of DTH sites for testing and controlling bovine tuberculosis and paratuberculosis.

Species' variation within the cellular infiltrate observed at the intradermal DTH testing site is an important factor to consider when planning research studies that evaluate the cell-mediate immune response at DTH sites. In human tuberculin skin testing, 75-90% of the mononuclear cells present are CD4+ T lymphocytes and monocytes [38]. In contrast, the DTH cellular infiltrate of mice is comprised largely of neutrophils [39]. Gulliver et al. demonstrated a predominance of a mixed mononuclear cell infiltrate present in sheep similar to the human tuberculin DTH infiltrate [40]. In this investigation, the sheep with less extensive intestinal lesions had elevated levels of IFNγ in the serum and a greater degree of cellular infiltrate within the DTH site. However, sheep with more severe lesions, including lesions located outside of the gastrointestinal tract, had an absent to small cellular infiltrate at the DTH site, high serum anti-MAP antibody, and high serum IFNγ levels [40]. This pattern observed in sheep within the DTH sites and intestinal tissues is consistent with the well-

described Th1 to Th2 transition in which IFNγ levels drop with an increase in humoral antibody response and lesion progression.

Review of the current literature revealed histological analysis and cell identification in DTH sites of cattle has not been extensively evaluated. In an investigation with Holstein calves, Plattner et al. compared the skin thickness of DTH sites between naïve, MAP-infected, and MAP-vaccinated cattle. As expected, MAP-infected and MAP-vaccinated calves had significantly thicker DTH sites. In comparison to the ovine study described above that histologically evaluated DTH sites, this investigation in cattle by Plattner et al. collected cellular infiltrate via retrieval and analysis of a biopolymer containing MAP antigen placed subcutaneously for 48 hours; in theory, this antigen and biopolymer study mimicked a DTH skin test. Using flow cytometry, the phenotypes of the T cells retrieved were identified; it was found that γδ T cells were observed at greater frequency 7 days post infection versus 30 days post infection, in which CD4+ T cells were the predominant cell type. IFNγ was also measured and levels were higher in polymer containing MAP compared to control sites. IFNγ was also higher at polymer sites 30 days post infection compared to 7 days post infection [41]. These findings may indicate that γδ T cells are initial responders in bovine DTH sites and generate significant amounts of IFNγ for activation of CD4+ T cells, dendritic cells, and macrophages. The described species' variation of cellular infiltrate observed in DTH sites is important to note when using different models for MAP infection/vaccination studies.

IFNγ and IL-10: Cytokines involved in MAP immune response

As discussed above, the role of the immune response, particularly the Th1/Th2 responses, during initial infection and disease progression is highly complex. A better understanding of

the definitive markers of protective immunity, active infection, and latent, subclinical infection is essential to advance vaccine development as well as prevent, diagnose, and control disease. The Th1 and Th2 responses are often measured by the cytokines they are known to produce, such as IFNγ and IL-10 respectively. Classical Th1, pro-inflammatory cytokines include IL-1α, IL-1β, IL-6 IL-12, IL-23, IL-17, TNFα, and IFNγ[42]. IFNγ is a well-known, well-studied, Th1 cytokine of mycobacterial infections and was the cytokine used most extensively in these chapters to evaluate the Th1 response. The production of Th2 cytokines is associated with a humoral response in which proliferation of B cells and inhibition of the Th1 response is observed. Classical Th2, anti-inflammatory cytokines include IL-4, IL-5, and IL-10 [43]. IL-10 and its role in the MAP immune response is an increasingly popular area of investigation; IL-10 was the anti-inflammatory cytokine used to evaluate Th2 responses in chapters 2-4.

IFNγ is a Type II interferon secreted by CD4+ Th1 cells, CD8+ T cells, γδ T cells, APCs, and natural killer cells [44]. In more recent investigations, other cells including B cells, NKT cells, and neutrophils have been shown to secret IFNγ [45-48]. IFNγ is a crucial player in the Th1 response and protection against intracellular pathogens such as mycobacteria. IFNγ is known to promote innate cell-mediate immunity by activation of NK cell effector functions [49]. Through T cell:APC interactions, IFNγ functions to promote specific cytotoxic immunity. Finally, one of the more important functions of IFNγ includes macrophage activation. Kamijo et al. demonstrated the significance of IFNγ in mycobacterial infection using mice lacking the IFNγ receptor. Mice with a disruption of the IFNγ receptor gene and wild-type mice were inoculated with *M. bovis*; infection with *M. bovis* was only lethal for the mice without a functional IFNγ receptor [50]. Both mice and humans

demonstrate a higher susceptibility to infection with intracellular pathogens and non-pathogenic mycobacteria, respectively, when mutated IFNγ genes are present [24, 51].

IL-10 is an anti-inflammatory cytokine also known as cytokine synthesis inhibitory factor (CSIF) [52]. Produced by macrophages, B cells, and T cells, IL-10 is best known for its strong anti-inflammatory activities [53]. The expression of IL-10 is crucial for controlling immune responses after infection. Knockout studies in mice with defective IL-10 demonstrated an uncontrolled level of IL-12 and IFNγ compared to wild-type mice; these IL-$10^{-/-}$ mice also exhibited a significant increase in the Th1 cell population in their colon mucosa. The findings of this study indicated the importance of IL-10 in regulating immune responses, particularly in the gastrointestinal tract [54]. Specific to mycobacteria, IL-10 has been shown to be released following phagocytosis of pathogenic *M. tuberculosis* [55]. Widdison et al. also showed a correlation between enhanced IL-10 levels and progression of *M. bovis* in cattle [56]. Several studies have looked at the effects of IL-10 suppression on IFNγ levels in antigen-stimulated peripheral blood from animals infected with different mycobacterial pathogens; the study results showed enhanced IFNγ levels in the whole-blood IFNγ assay when anti-IL-10 antibody was removed[57, 58].

Prior research of cytokine profiling in MAP infection has focused on peripheral blood immune responses, as well as sites of infection such as ileum and lymph node [15, 59]. There have been several papers focused on cytokine expression profiles in cattle, sheep, and goats [16, 60, 61]. One example is the extensive work by Coussens et al. and their examination of the gene expression of 13 different cytokines in PBMCs, intestinal lesions, and mesenteric lymph nodes of MAP-infected and naïve cattle. The findings in the PBMCs demonstrated that only IL-10 gene expression was increased with MAP antigen stimulation of the PBMCs

in subclinical cattle, and the levels of IFNγ, IL-1α, and IL-6 were reduced with MAP antigen stimulation of the PBMCs. These findings provided supportive evidence for the enhancement of anti-inflammatory cytokines and suppression of pro-inflammatory cytokines when cells were stimulated with MAP antigen. Ileal tissues exhibited greater expression of pro-inflammatory cytokines in infected compared to naïve cattle [15]. This pro-inflammatory response observed in the tissues, along with the Th1 suppression observed in stimulated PBMC of infected cattle, is a well-known finding in mycobacterial research [15, 16, 58, 60-62].

The cytokine expression profiles in PBMCs and tissue samples provide beneficial information on mycobacterial immune responses and diagnostic markers at the systemic and gastrointestinal level. However, minimal current literature exists on the immune responses, cellular infiltrate, and cytokine profile within the intradermal testing site in cattle. Human studies have used immunohistochemical evaluation to quantify the cells containing different cytokines at several time points post-PPD intradermal injection in Bacillus Calmette–Guérin (BCG)-vaccinated individuals. Cells containing IL-1α, IL-1β, IL-6, IFNγ, and TNFα were observed as early at 6 hours after PPD injection. IFNγ was observed in the highest frequency of cells and 33% of cells were IFNγ positive at 48 hours; however, by 7 days post intradermal injection IFNγ had dropped to 3% of total cells. Using dual fluorescent labeling, IFNγ positive cells were shown to be CD3+ T cells compared to the TNF-a, IL-l and IL-6 positive cells which were CD68+ macrophages [63]. A better understanding of the expression of *in vivo* cytokines in a MAP-induced DTH reaction may provide helpful information about the cell-mediated immune response observed in vaccinated and naïve cattle.

MAP vaccination: Introduction

The current strategies to control MAP infection rely on prevention of new infections, particularly during the neonatal period [1]. These control strategies include management practices to improve calving hygiene and rearing, test-and-cull strategies, and vaccination. More hygienic calving practices and test-and cull-strategies are known to reduce MAP infection in herds; however, both of these strategies require long-term management and producer dedication before economic benefits are observed [64]. The economic costs of test and cull strategies have been shown to be higher than their financial benefits [65]. Vaccination is known to decrease MAP fecal shedding and reduce numbers of clinically affected cattle [66]. Field investigations that have evaluated MAP vaccination on MAP-infected herds found that vaccination strategies reduced fecal shedding of MAP and the number of cows with clinical disease in the herd [67]. One field study also found MAP vaccination was cost-efficient in herds with a high prevalence of infection [68]. However, most of the research on cost to benefit analysis of MAP vaccination has been based on mathematical models and hypothetical vaccines with superior protection [64]. Mathematical models are often used because the long incubation period of MAP hinders a controlled clinical trial [69]. A critical need exists for a more economical strategy to prevent and control Johne's disease in cattle herds. MAP vaccination has the potential to reduce economic losses and control MAP infection; however, a more efficient model to screen future vaccine candidates is needed. The focus of the research in chapters 2-4 is based on this need and includes the development and evaluation of a potential device used for *in vivo* CMI analysis and vaccine candidate screening.

Current and future vaccines

The current approved vaccine for MAP in the United States is the killed bacterin product, Mycopar®, available on a conditional basis with state veterinarian approval. Mycopar® is made by Boehringer Ingelheim Vetmedica Inc. [70]. The complication of this vaccine is the potential for MAP vaccination to cause a positive *M. bovis* intradermal test further requiring the effort and expensive of a comparative cervical test. Therefore, it has been hypothesized that widespread vaccination of MAP could largely affect the *M. bovis* surveillance program [64]. Silirum®, manufactured by Zoetis, and Gudair®, produced by CZ Veterinaria, are two commercially available, inactivated, whole-cell vaccine preparations; currently, these vaccines are not available in the United States [71].

Despite the current limitations and drawbacks, vaccination still holds the potential to control or eradicate Johne's disease. A great need exists for the development of new vaccine candidates with improved efficacy; vaccines are needed that prevent bacterial transmission and fecal shedding. Also, an ideal vaccine candidate would not form an injection site granuloma that is currently observed following MAP vaccination [72-74]. It would be beneficial to produce future candidates that demonstrate immune protection specific to the MAP immune response without the current cross-reactivity that interferes with *M. bovis* control and surveillance.

Research indicates that subunit vaccines may prove to have fewer drawbacks than the currently available whole-cell vaccines. Recent studies of subunit vaccines showed a reduction in granuloma formation with injection; however, improvements in protection against MAP infection were not observed in these murine, bovine, or caprine models [75-77]. Koets et al. demonstrated successful use of recombinant MAP Hsp70 as a subunit vaccine

against bovine paratuberculosis by reducing shedding of bacteria in feces during the first two years following experimental infection [75]. Santema et al. has shown that the HSp70/DDA subunit vaccine does not interfere with current *M. bovis* diagnostic assays. In addition, Santema et al. discovered that by performing an extra step during the ELISA assay, distinction between infected cattle and cattle vaccinated with Hsp70/DDA can be achieved through this assay; simple removal of the Hsp70/DDA vaccination induced antibodies will reestablish the specificity of the ELISA test [78]. The absence of granuloma formation and lack of *M. bovis* test interference are significant benefits to subunit vaccines; however, these new candidates have not yet demonstrated an improvement in MAP protection over the commercially available vaccines.

In addition to the subunit vaccine studies, there has been an increased research spike in the development of live-attenuated vaccines. Advantages of this type of vaccine include strong stimulation of innate and adaptive immune responses and low cost of manufacturing [3]. In order to attenuate the virulence of MAP for use in vaccines, various mutants have been developed for investigation of their efficacy at providing protection against infection. In previous genome studies, Hsu et al. identified *sigH* as a mycobacterial gene regulator in MAP [79]. Ghosh et al. utilized this gene identification to evaluate the protective efficacy of a vaccine candidate with a *sigH* mutant compared to a wild-type MAP K10 strain. Bacterial burden was greatly reduced and IFNγ levels significantly elevated in the *sigH* mutant vaccine candidates. The *sigH* mutant vaccine study demonstrated superior protection in comparison to the wild-type strain [80]. In addition to the *sigH* mutant study by Ghosh et al., other researchers have also used the murine model as a screening test for various MAP vaccine mutants [81, 82]. For instance, Settles et al. used the murine model to investigate two vaccine

mutants, pgs1360 and pgs3965, that contained mutations in separate genes. After MAP challenge, both vaccine candidates showed reduced bacterial loads within organs compared to control mice [82]. Shippy et al. further evaluated the *sigH* and *pgs* mutants in a caprine model. Both mutant vaccines demonstrated reduced lesions and low bacterial load within intestinal tissues compared to naïve goats and goats vaccinated with an inactivated vaccine. One of the mutant vaccines was shown to eliminate fecal shedding, which would be highly beneficial to the control and eradication of MAP infection [6]. In summary, the development of new vaccine candidates is promising for future use in Johne's disease control programs; however, this area of research is slow to progress, largely due to the financial constraints and lengthy duration of vaccine testing models.

Animal models and vaccine candidate screening

Multiple factors hinder current investigative efforts to improve the efficacy of MAP vaccines. For example, high costs are incurred during vaccine trials using natural hosts such as sheep, goats, cattle, and deer. Secondly, the characteristic long latent period of infection makes screening vaccine candidates in natural hosts an extensive and long process. Finally, the lack of standardized animal challenge models makes comparison of new candidates in different vaccine trials highly problematic [71]. Fortunately, efforts toward standardizing animal challenge models and vaccine trials for MAP have been implemented, benefiting the research and development of novel vaccines. Supported by the Johne's Disease Integrated Project (JDIP), a committee of MAP researchers was formed called the JDIP Animal Model Standardization Committee (AMSC), and these 16 members collaborated to form guidelines and parameters for vaccine efficacy studies in models of different species. Based on expert

consensus, a list of parameters for each MAP host model was formulated [83]. In a paper by Bannantine et al., the standardized testing parameters and models were used to evaluate 22 vaccine candidates developed across the United States and New Zealand. The studies utilized the JDIP Animal Model Standardization Committee guidelines to establish a 3-phase trial: Phase 1 evaluated survival in bovine macrophages, and the mutants that showed a reduced pathogenicity or vitality advanced to the Phase II mouse trial. In Phase II, eight attenuated vaccines were used in a murine model and evaluated for bacterial load in tissues at varying time points after MAP challenge. In the murine model, 5 of the 8 candidates showed bacteria that did not survive in the liver and spleen. Finally, Phase III was conducted in goat kids. In this goat model, a reduction in incidence and severity of infection was observed in some vaccine strains; however, none of the vaccines eliminated fecal shedding [71, 81]. The results of the study indicate that the use of the 3-phase, macrophage to mouse to ruminant platform may need adjustments, particularly in the macrophage and mouse models where evidence of attenuation *in vitro* and within an unnatural host, respectively, does not appear to predict natural host protection [71, 81, 84]. In summary, cell culture and mouse trials may not be ideal models for screening vaccine candidates to show protective immunity in a natural host. However, the efforts by the JDIP Model Standardization Committee to define parameters and standardize natural host models will prove beneficial for vaccine candidate comparison in trials across different research laboratories. Future development of approaches to make vaccine trials more efficient and economical using natural host models are necessary to facilitate the research being generated on novel MAP vaccine candidates.

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CHAPTER 2. DEVELOPMENT OF A REMOVABLE SUBCUTANEOUS IMPLANT FOR IN VIVO DETECTION OF CELL-MEDIATED IMMUNE RESPONSES IN CALVES VACCINATED FOR MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS

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Abstract

Improved vaccine candidates are increasingly sought for control and ultimate elimination of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from cattle herds. A current obstacle in MAP vaccine development is the expense and lengthy incubation period associated with vaccine-challenge experiments in the bovine model. To aid in the development of successful MAP vaccines, an initial screening test that evaluates the *in vivo* immune response after MAP challenge would provide knowledge of the vaccine's efficacy in a shortened time frame. In this study, we discuss the development of a device placed within the subcutaneous tissue for *in vivo* collection of antigen-specific cytokines and cellular infiltrate that can be measured *ex vivo* upon retrieval. We assessed histologic sections and measured cytokine levels in implant collagen retrieved from the neck subcutis of naïve and MAP-vaccinated cattle. We found the collagen containing MAP antigen showed a markedly

increased cellular infiltrate similar to the increase in cellular infiltrate observed with delayed-type hypersensitivity (DTH) responses. Detectable concentrations of IL-10 and IFN-γ were extracted from the retrieved collagen and IFN-γ was significantly higher in the collagen containing MAP antigen implanted into MAP-vaccinated calves.

Introduction

Mycobacterium avium subspecies *paratuberculosis (*MAP) is the etiologic agent of Johne's disease, a chronic, granulomatous enteritis of ruminants often resulting in progressive diarrhea, weight loss, and loss of conditioning [1]. Neonates are most susceptible to infection, and the fecal-oral route from cow to calf is considered the primary mode of transmission [2]. Following MAP infection of calves, a prolonged incubation period (>2yrs) develops that is characterized initially by variable cell-mediated (Th1-type) immunity [3, 4]. During this stage of infection, clinical signs and bacterial shedding do not occur; detection of these animals is therefore difficult. A proportion of infected cattle will eventually progress to clinical disease where the Th1 response wanes and an ineffective Th2 response develops [2, 3, 5, 6]. Treatment is not typically an option due to the high cost of antimycobacterial therapeutic agents combined with the high doses required for long treatment periods.

Due to difficulty in identifying MAP-infected animals, vaccination is increasingly looked to for control and elimination of MAP from cattle herds. The current commercially available vaccines against Johne's Disease, Mycopar® (Boehringer Ingelheim Vetmedica, Inc.), Silirium® Vaccine and Gudair® Vaccine (Zoetis AU), can delay and decrease clinical disease; however, vaccination does not prevent fecal shedding and transmission [7, 8]. The ideal vaccine for MAP infection would induce a sustained cell-mediated response that

prevents bacterial shedding and disease progression [4]. Development of new vaccines against paratuberculosis is an area of active research with protein subunit, DNA, and attenuated live vaccines being investigated as potential candidates [4, 7, 9]. An obstacle to development of new effective vaccines is the difficulty in testing vaccine candidates. This is due to the long incubation period following infection, which makes standard vaccine-challenge studies time-consuming and expensive. Moreover, alternative models including rodents and cell culture systems do not accurately predict the bovine immune response to vaccines [4, 9].

Detection of cell-mediated immune responses is a central component in testing new vaccines against mycobacterial infections. Methods for detection of cell-mediated immune responses in cattle include a delayed-type hypersensitivity (DTH) intradermal skin test and the interferon gamma (IFN-γ) release assay [3, 10, 11]. In both of these assays, there can be limited specificity due to antigen cross reactivity with nonpathogenic mycobacteria. The skin test provides a yes/no answer to the presence of a cell-mediated immune response, but intermediate results are common and difficult to interpret. The IFN-y release assay will allow for quantification of a single cytokine. However, the accuracy of the assay can be reduced by multiple factors including variation of the antigen preparations (PPD), loss of IFN-y signal during transit from the field to the lab, and artifacts induced from *ex vivo* culture conditions required for antigen stimulation.

Our laboratory set out to improve detection of cell-mediated immune responses to MAP vaccines in calves. Our strategy was to develop a platform that directly measures cell-mediated immunity within the animal. This platform uses a removable subcutaneous implant that houses MAP antigens. The cellular and cytokine responses to the antigens are retained

within the implant, and are assessed following implant removal. The rational for this approach is that it will measure the immune response at the actual site of antigen exposure in the living animal, thereby reflecting the true *in vivo* immune response. In this manuscript we describe the development and use of this assay. We demonstrate differential immune cell infiltrates and cytokine production in MAP vaccinated calves using the removable implant and compare this to a standard IFN-γ release assay.

Theory

The overall principle of this method is to measure immune variables associated with the delayed-type hypersensitivity (DTH) response within the subcutis of calves vaccinated for MAP. Our platform is a cell-permeable implant that contains a collagen-based polymer admixed with MAP antigens. The implant is placed in the subcutis for 48 hours, similar to a standard DTH skin test. The implant creates a self-contained environment for the immune response that retains recruited immune cells and secreted cytokines that, upon removal, can be measured in the laboratory using the methods outlined below. We expect that measuring multiple immune variables *in vivo* will enhance the ability to assess an animals' response to vaccination.

Materials and Methods

Animals and vaccination

Four Holstein bull calves that were approximately 250-300 pounds were procured by Laboratory Animal Resources at Iowa State University in Ames, Iowa. Calves were housed in an outdoor research facility and divided randomly into the following two groups: naïve

(n=2) and MAP vaccinated (n=2). Vaccinated animals were vaccinated in the ventral neck region with Mycopar®, a whole cell inactivated bacterin (Boehringer Ingelheim Vetmedica, Inc., Saint Joseph, Missouri), per the manufacturer's instructions. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA, USA).

PureCol® *EZ* Gel and MAP Antigen

The polymer used in this experiment is a Type I bovine collagen (*PureCol*® *EZ* Gel, Advanced BioMatrix, San Diego, CA). *PureCol*® *EZ* Gel is a commercially available, pre-formulated, purified Type I bovine collagen at 5mg/ml concentration that forms a firm gel at 37°C. The collagen contains CMEM/F-12 medium with L-glutamine and L-alanine-L-glutamine. *PureCol*® *EZ* Gel (hereafter referred to as collagen) was stored at 4°C in liquid form. A commercial preparation *Mycobacterium paratuberculosis* purified protein derivative johnin (PPD-J) intradermic tuberculin (NVSL, Ames, IA, USA) was used for this study and stored at 4°C. Johnin PPD is a solution of phenolized phosphate buffer and tuberculoprotein at a concentration of 1.0mg/ml. Prior to implantation, collagen containing 30 percent PPD-J or an equal amount of sterile saline, as a control, was loaded into the mesh lining within the cylinder of the implant. Implants were then incubated at 37°C for at least one hour allowing a firm gel to form.

Implantation procedure

Calves were restrained with the use of halter, chute, and an adjustable head gate. The right and left lateral necks were sterilely prepped and 3ml of 10mg/ml lidocaine was

dispersed into a localized area of approximately 4cm by 4cm. A 0.5cm incision was made via scalpel blade followed by insertion of the implant using the implantation device leaving the cylindrical portion of the implant in the subcutaneous tissues with the retrieval pin remaining external. To adequately stabilize the device, a single suture was placed through the epidermis and around the retrieval pin. Two implants were placed in each animal; the first, a control implant, containing only collagen and a second implant containing collagen and MAP antigen. The implants remained in the calves for a duration of 48 hours.

Implant retrieval and collagen processing

Implants were removed via retraction on the retrieval pin and transported back to the laboratory. A single interrupted suture was placed in the skin to close each implant site. The nylon mesh and associated collagen plug were removed from the stainless-steel housing of each implant. The collagen was then removed from the nylon mesh lining. For histopathology, a section of collagen was placed in 10 percent neutral buffered formalin and processed routinely for hematoxylin and eosin (H&E) staining.

To quantify the cellular density within the fixed collagen sections, total nucleated cell counts (TNCC) were performed at high magnification (100x objective); each group (n=2) had a total of 10 fields counted (5 fields per individual implant). For cytokine analysis, the remaining collagen was cooled on ice and thoroughly minced in complete RPMI media (cRPMI, Life Technologies, Grand Island, NY, USA). The supernatant was then collected and stored at -20°C for future cytokine analysis (n=2 implants per group).

Blood collection and cell culture

Four blood samples were collected from naïve (n=2) and MAP vaccinated (n=2) calves. Ten milliliters of whole blood was collected in acid citrate dextrose via jugular venipuncture and peripheral blood mononuclear cells (PBMC) were isolated using buffy coat density centrifugation. PBMC were suspended in cRPMI medium supplemented with 10% (v/v) FBS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 2% MEM essential amino acids, 0.1% penicillin-streptomycin, and 0.004% 2ME (all purchased from Life Technologies). Cells were diluted to a final concentration of $5x10^6$ cells/well in a 96-well plate and stimulated with Concavalin A (Sigma-Aldrich, St. Louis, MO, USA) at 2ug/ml, Johnin PPD (National Veterinary Services Laboratory, Ames, IA, USA) at 10ug/ml, or cRPMI medium for 24 hours at 37°C and 5% CO_2. Supernatants were collected and placed at -20°C for future cytokine analysis.

Luminex® multiplex assays

Supernatants from the implant collagen and MAP antigen-stimulated PBMC were incubated with magnetic beads in a 96-well plate at room temperature for two hours. Magnetic beads (MC10062-01, MC10026-01, Bio-Rad Laboratories Inc, Hercules, CA, USA) were pre-conjugated with IL-10 and IFN-γ capture antibody (MCA2110, MCA2112, Bio-Rad). Biotin-labeled IL-10 and IFN-γ detection antibodies (MCA1783B, MCA2111B, Bio-Rad) were added and incubated for one hour followed by a 30-minute incubation with eBioscience™ Streptavidin PE (Invitrogen, Thermo Fisher Scientific Inc, USA). The mean fluorescence intensity and bead classification was determined via Bio-Plex®200 reader (Bio-Rad) and compared to a standard curve to derive cytokine concentrations of each individual

sample. The following proteins were used to create a standard curve: recombinant bovine IFN-γ and recombinant bovineIL-10 (PBP007A, PBP016A, Bio-Rad).

Graphs and analysis

Graphs were made using GraphPad Prism 7 (La Jolla, CA). All data are shown as mean + standard deviation (SD). Normality tests (D'Agostino-Pearson omnibus normality test) were performed on the datasets, and in some instances, the data did not have a normal distribution; therefore, nonparametric tests were used for all analyses. Differences between MAP antigen usage for TNCC (Fig. 6) and cytokine levels in collagen (Fig. 7) were assessed using Kruskal-Wallis test for multiple comparisons. Due to small experimental group size (n=2), statistical analysis could not be performed on cytokine levels measured from stimulated PBMC (Fig. 8).

Results and discussion

Implant design

The goal of this study was to create a removable implant that would be a self-contained environment for cell-mediated immune responses to occur within the subcutaneous tissue of cattle. Immune mediators contained within the implant would then be measured after it was removed from the animal. In this way we would measure *in vivo* immune responses at the sites of vaccination. We designed an implant based on our previous work directly placing Matrigel^{TM} mixed with MAP antigens into the subcutaneous tissue of calves [12]. In these earlier studies we were able to detect cell-mediated immune responses within the Matrigel^{TM} after its removal from the calf. However, recovering Matrigel^{TM} required

surgical biopsy. We needed to create an implant that could be rapidly placed and removed from the animal.

The designed implant device **(Fig. 1)** consists of an outer stainless-steel housing that is 7cm x 4mm. At one end of this housing there is a solid 3.5cm solid retrieval pin that attaches to a 4.5cm hollow cylinder. Two rectangular windows are present along the length of the hollow cylinder portion of the implant **(Figure 1, insert**). A nylon mesh screen, with 30um pore size, lines the interior of the cylindrical portion of the implant and communicates with the exterior of the implant via the rectangular windows. The nylon mesh screen surrounds a core of up to 350ul of collagen and directly opposes the two windows of the cylinder. The collagen contains the MAP antigen (here we used PPD-J). The implant is designed to allow recruitment and migration of immune cells across the nylon mesh into the collagen core where the MAP antigen is located. An implantation device was developed for this implant **(Fig. 2),** which places the implant beneath the skin into the subcutaneous tissue. For this study, the pin was left on the exterior of the animal (**Fig. 3**) and secured with single suture. The implant was removed by gentle retraction on the retrieval pin. In subsequent studies, the implant was buried in the subcutis and the incision closed with suture (not shown).

We have used two types of collagen in the implant, PureCol® EZ Gel and BD Matrigel™ Matrix and have measured cytokines and cellular infiltrates from both. BD Matrigel™ Matrix, is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. PureCol® EZ Gel is a purified Type I collagen from a bovine source. Matrigel™ is thermoreversible and after cooling the gel readily returns to a liquid state. This facilitates recovery of recruited immune cells and

proteins from the implant. However, cattle can develop an immune response to the mouse proteins in the Matrigel™ with repeated use. PureCol® EZ is purified bovine collagen that is non-immunogenic and is well suited for repeated use in longitudinal studies. The PureCol® EZ is not thermoreversible and requires additional processing (mincing) to recover intact cells and cytokines. PureCol® EZ and Matrigel™ can be fixed and processed as any solid tissue for histopathology. We have found that the antigen (PPD-J) mixes well in both types of collagen. As a negative control we use an implant with collagen but no antigen for detection of baseline immune responses to the collagen alone. The data shown in this manuscript are from implants containing PureCol® EZ.

Implant placement into MAP vaccinated calves

We used the implant to detect cell-mediated immune responses in Mycopar®-vaccinated calves. MAP vaccination with this product leads to a strong cell-mediated immune response in calves 1-2 weeks post vaccination [13]. One group of calves was vaccinated with Mycopar® and a second group was given a sham vaccine of saline. Two weeks post-injection, vaccinated calves developed an anticipated firm swelling at the injection site (vaccine granuloma) [14]. Two weeks post-vaccination two implants were placed in each calf, one with collagen containing MAP antigen and one with collagen only. Calves were restrained in a chute with a head gate for implant placement and removal. With the aid of lidocaine local analgesia, we did not experience any difficulty placing the implants. The implantation process, including sterile preparation of the neck, took approximately 10-15 minutes per calf. The implants remained in the subcutis for 48 hours and were then removed. All implants were recovered and calves did not demonstrate any adverse effects. After

implant removal, the steel housing was separated from the nylon and collagen core. The nylon screen was then separated from the collagen (**Fig. 4**). **Figure 4A** demonstrates the nylon mesh surrounding the collagen after removal from the steel housing. **Figure 4B** demonstrates opening of the nylon screen and removal of the collagen. The collagen was then split into two equal halves; the first half being processed for histopathology and the second for cytokine analysis.

Histological evaluation of immune cell recruitment into the implant

After removing the collagen **(Figure 4B**) from the implant it is processed to slides using the same procedures as any solid tissue. Histologic sections of the collagen recovered from the calves are shown in **Figure 5**. These images demonstrate that when antigen is not present in the collagen, regardless of vaccination status, there is minimal cellular infiltrate into the collagen (**Fig. 5 A, C**). In contrast, in sections where MAP antigen (PPD-J) was included in the collagen, a greater cellular infiltrate was observed (**Fig. 5 B, D**). The density of recruited cells is significantly higher **(Fig. 6)** in implants containing MAP antigen that were placed and retrieved from vaccinated calves. This demonstrates antigen dependent immune cell recruitment to the implant and immune cell migration through the nylon mesh into the collagen core. Based on morphology, this cellular infiltrate consisted of numerous neutrophils intermixed with fewer lymphocytes and macrophages.

We hypothesized that the infiltrating cells would be in similar proportions as a delayed-type hypersensitivity (DTH) reaction to standard mycobacterial skin testing with lymphocytes and macrophages being the dominate cell types. Skin testing is an *in vivo* test commonly used to identify cell-mediated immune responses to multiple mycobacterial

infections including *M. tuberculosis*, *M. bovis*, and *M. leprae*. The skin test uses a purified bacterial protein preparation (PPD) as antigen. PPD is injected directly into the dermis, and sensitized animals will develop a DTH reaction that is maximal 48-72 hours post injection. PPD preparations vary according to the mycobacterial species that is being tested. Skin swelling size is the sole parameter measured, but histopathology of the injection site has been evaluated. Previous investigators have demonstrated that the early microscopic features of the DTH in skin tested *M. bovis* infected cattle include a cellular infiltrate into the dermis that is predominately comprised of neutrophils and mononuclear cells, and as the reaction progresses the degree of mononuclear cells increases [15]. While neutrophils were the main cell detected in the collagen in this study, it is possible that lengthening the duration of time in which the implant device is placed from 48 to 72 hours would increase the percentage of mononuclear cells. In future studies we will use immunohistochemistry for lymphocyte and macrophage markers to further quantify the mononuclear cell infiltrate.

Cytokine analysis from implants and from antigen stimulated peripheral blood mononuclear cells

We next measured cytokine concentrations in the implants recovered from naïve and vaccinated calves. After implant removal, the collagen was cooled, minced, washed and the supernatants collected. A bead-based multi-analyte profiling assay (Luminex®) was used to measure cytokines within the collagen. We hypothesize that measuring multiple cytokines will provide more information on the immune response over single analyte tests, such as the IFN-γ release assay. For this study we selected IFN-γ and IL-10. IFN-γ is a Th1 cytokine and a central driver of innate and adaptive immunity against intracellular pathogens [16].

Production of IFN-γ occurs early in the course of MAP infection and MAP vaccination. In MAP-vaccinated cattle, it has been shown that MAP specific IFN-γ production by PBMC occurs by seven days post-vaccination and remains elevated for up to one year [13]. IL-10 is known to suppress macrophage activation and down-regulate inflammatory cytokines [17]. IL-10 has been shown to be suppressed in calves given a modified live MAP vaccine [18]. A ratio of IL-10 to IFN-γ has been used to characterize the immune response to mycobacterial infection in humans and correlates to severity of clinical disease [19].

Cytokines were quantified using standard curves for bovine recombinant IL-10 and IFN-γ. The highest concentration of IFN-γ ($P < 0.05$) was detected in collagen containing MAP antigen that had been placed in MAP vaccinated calves. IFN-γ was not detected in naïve calves or from implants without MAP antigen **(Fig. 7A)**. This data suggests that the implant is able to elicit antigen-dependent cytokine production within the animal. It also demonstrates that the collagen in the implant retains the generated cytokines, which can be measured after implant removal. IL-10 (**Fig. 7B)** was detected at higher concentrations than IFN-γ **(Fig. 7B** note y-axis scale difference**).** However, this was not antigen or vaccine-dependent. In future studies we will continue to assess cytokines that have been shown to be associated with immune responses to mycobacterial vaccination including TNFα, IL-17, IL-23, IL-6, IL-4, and IL-1β.

We compared the cytokine concentrations from our implants to cytokine release assays using PBMC collected from calves immediately prior to implanting. IFN-γ and IL-10 production were measured from cultured PBMC that were stimulated with PPD-J, media or Concanavalin A. As expected, only the PBMC from vaccinated calves showed IFN-γ production after stimulation with PPD-J **(Fig. 8A, B).** The pattern of cytokine production in

PBMC followed that from the implants where IFN-γ was detected only in implants with antigen placed in vaccinated animals. Differences in IL-10 concentrations from stimulated PBMC were not observed between the calves, similar to the implant cytokine data. This data demonstrates comparable results for cytokine production in the implant collagen and cytokine release assays, and provides proof of principle for using this implant to detect immune responses in vivo.

Conclusions

We have developed an implant that serves as a self-contained environment within the animal where immune responses to a given antigen occur. Parameters of this immune response can be measured upon implant removal. This study describes placement and recovery of implants from the subcutis of naïve and MAP-vaccinated cattle. We measured cellular recruitment into the collagen of the implant using histopathology, as well as cytokine concentrations in the collagen using a multiplex immunoassay method. We show increased immune cell infiltrate into the implants placed in MAP-vaccinated calves and a correlating increase in the concentration of IFN-γ. To our knowledge, this is the first MAP-specific *in vivo* measurement of IFN-γ. We also detected IL-10 in all implants, but neither antigen or vaccination status influenced IL-10 concentration. The advantage of this system is that multiple parameters can be evaluated simultaneously to more accurately assess the *in vivo* immune response. The parameters included in the assay can be tailored to each study and increase the versatility of the system.

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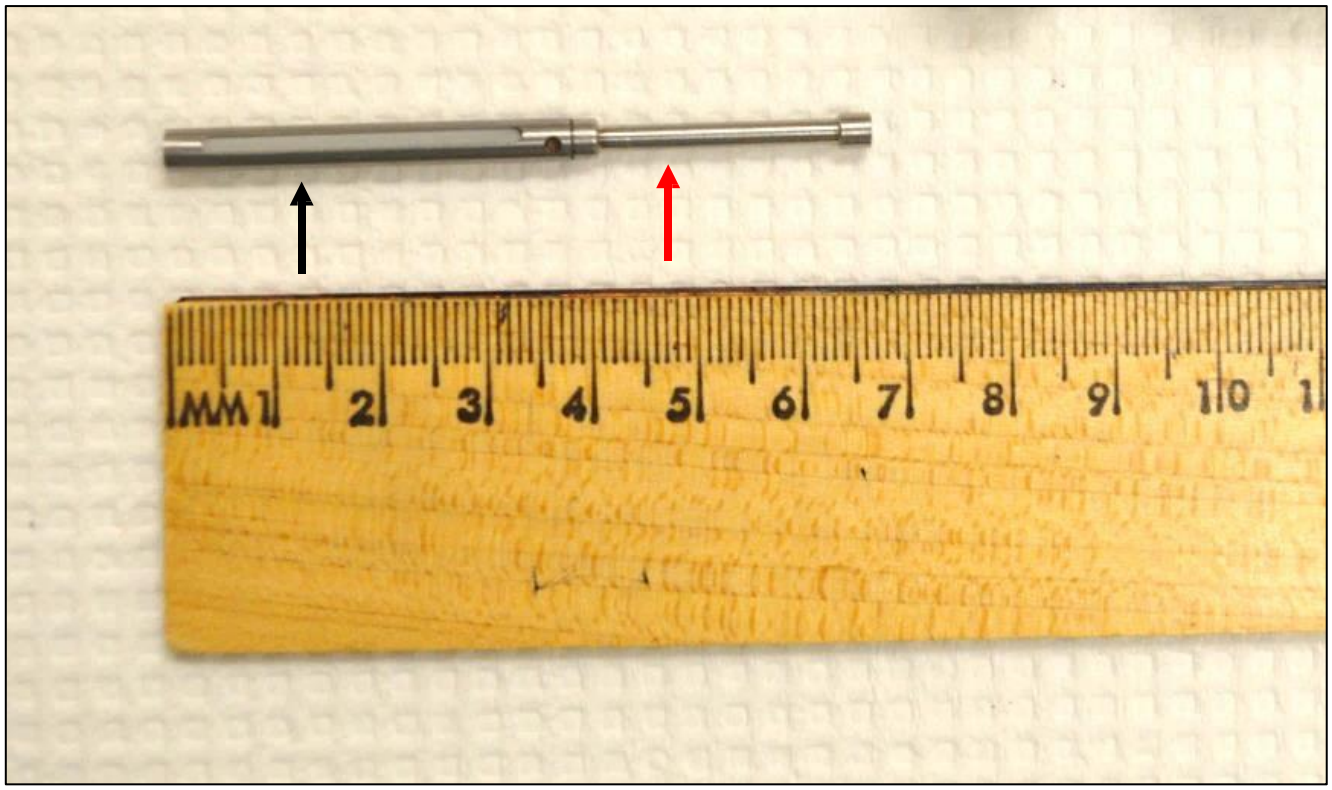

Figure 1: Implant Design. The implant holds 300ul of polymer. It has a window (insert) lined by a nylon mesh screen (black arrow). The implant is removed by retraction of a steel pin attached at one pole (red arrow).

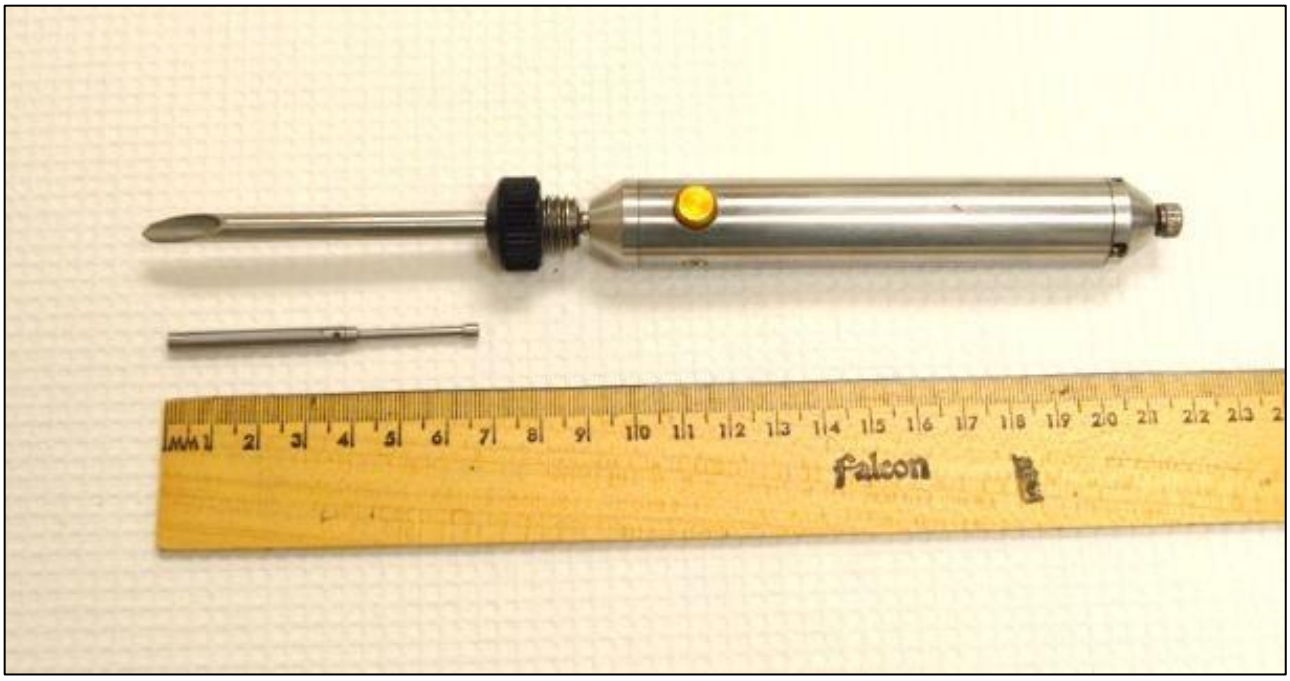

Figure 2: Implant placement device. This device is used to inject the implant into the subcutis.

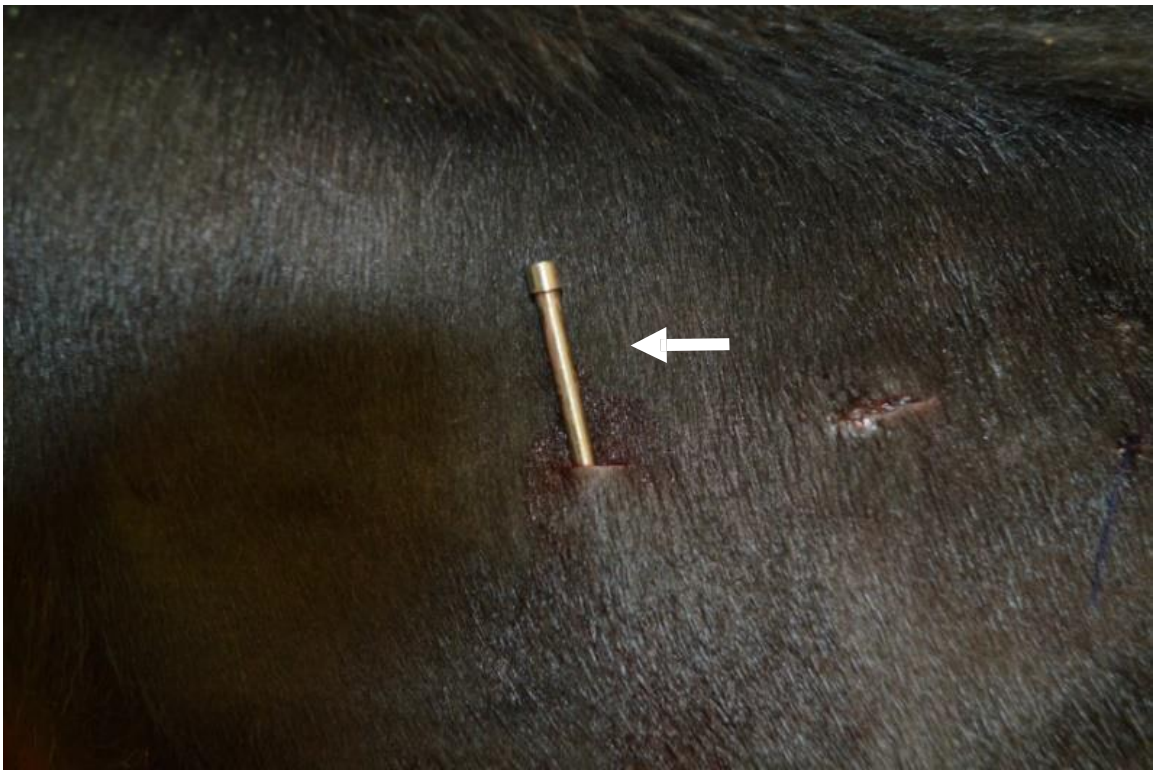

Figure 3: Implant placed in the calf. The removal pin (white arrow) in this experiment was left on the exterior of the animal and would be secured with a suture. The housing containing the collagen polymer is within the subcutis.

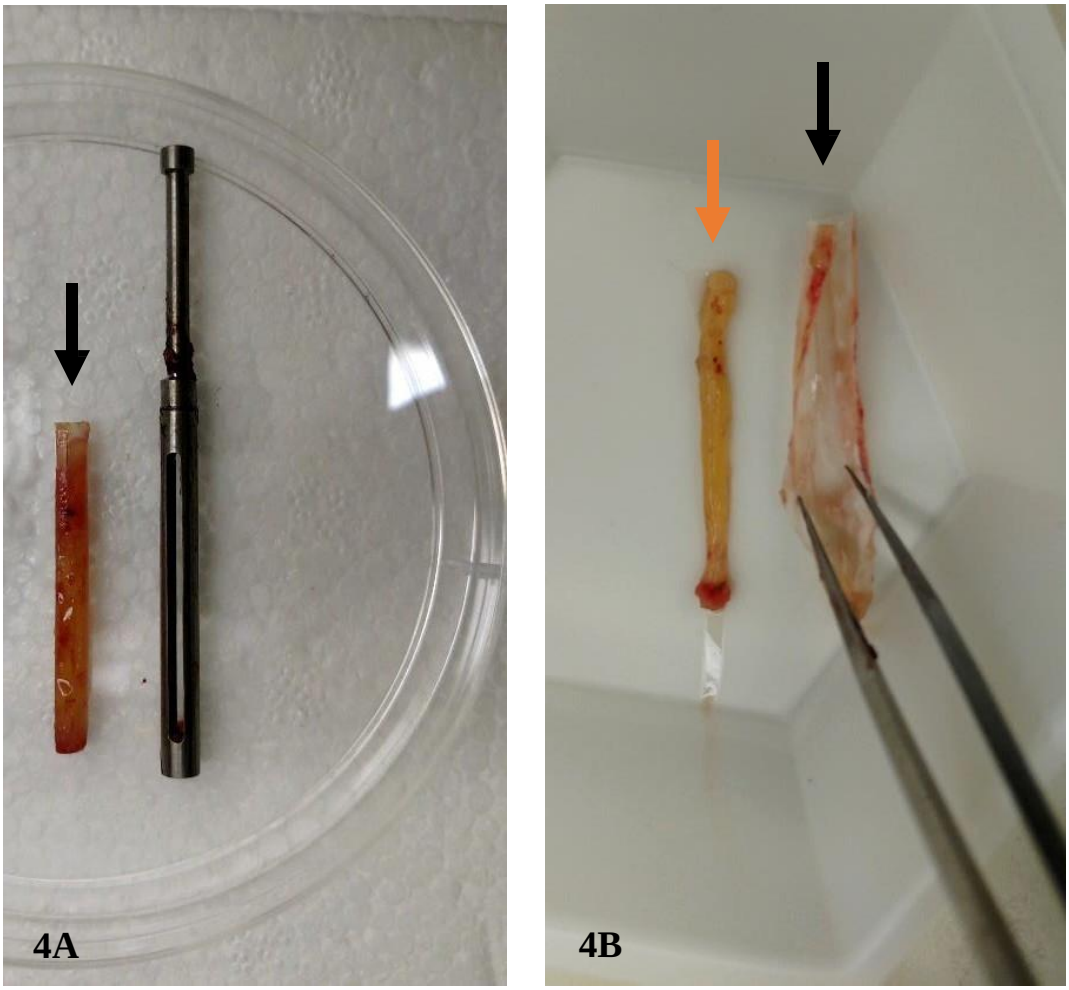

Figure 4: Removal of polymer from implant. 4A – the nylon mesh (black arrows), which surrounds the polymer is pulled from the end of the implant. 4B – the nylon mesh is opened and the polymer (orange arrow) is removed for further processing.

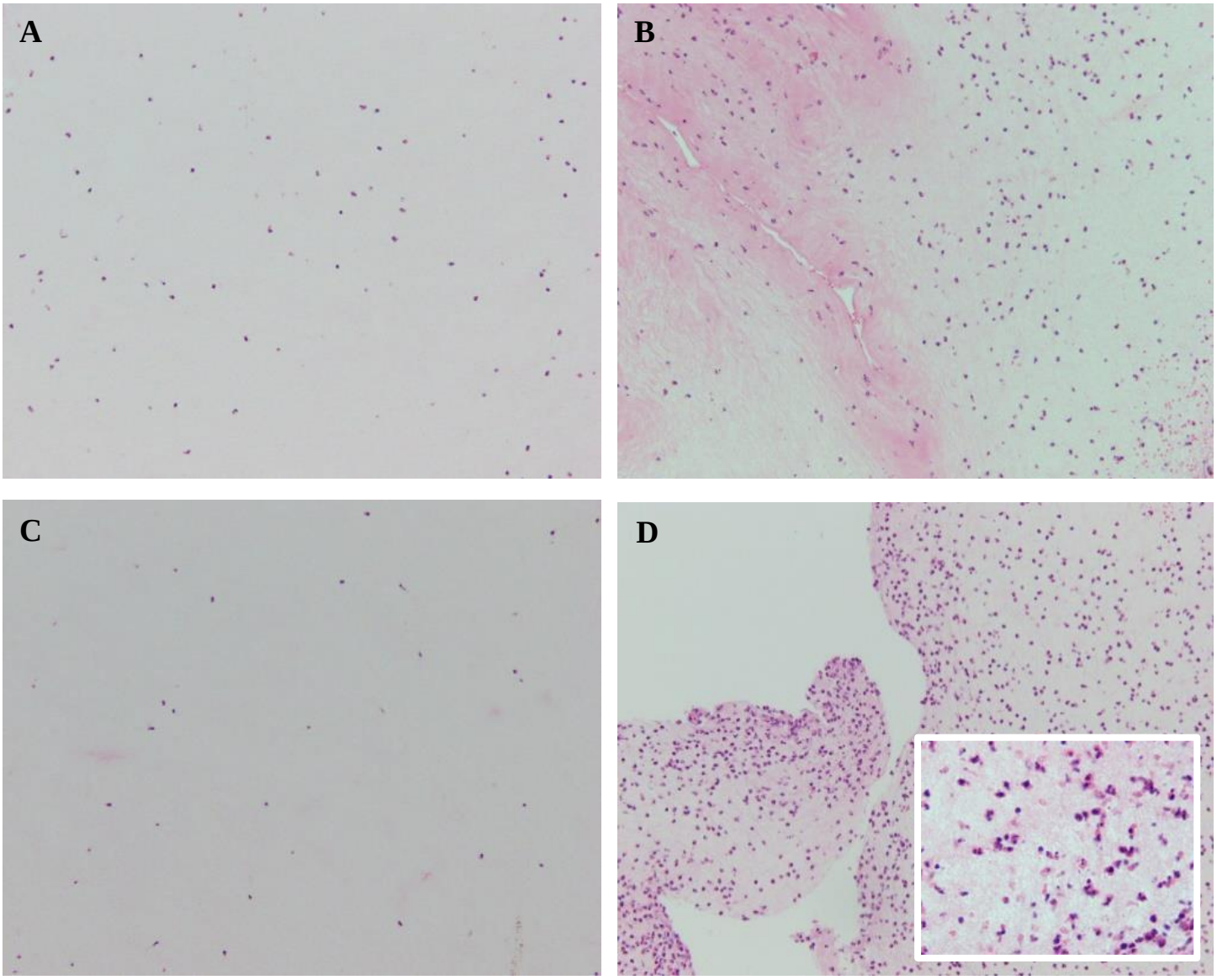

Figure 5 (20x): Immune cell recruitment into collagen. A – collagen with no MAP antigen recovered from a naive calf. B – collagen with MAP antigen recovered from a naive calf. C – collagen with no MAP antigen recovered from a vaccinated calf (insert, 100x). D – collagen with MAP antigen recovered from a vaccinated calf. These images demonstrate that cellular recruitment was strongest in the collagen recovered from vaccinated calves where MAP antigen was included in the collagen.

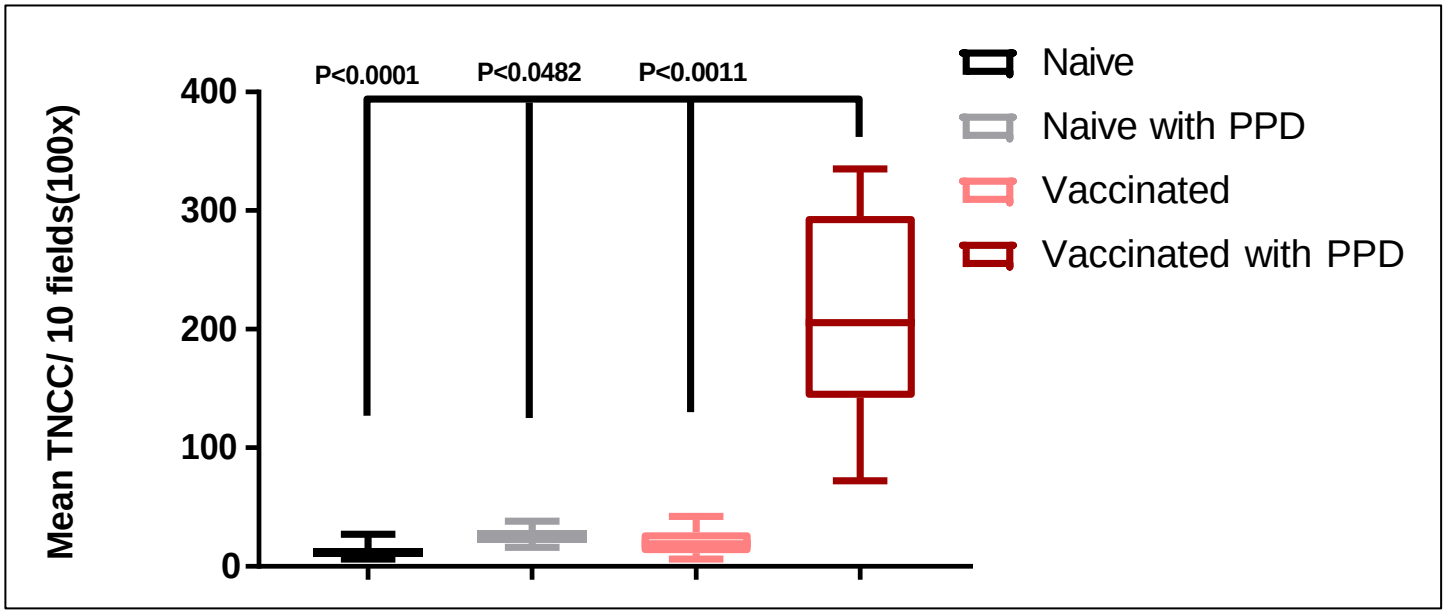

Figure 6: Number of cells observed within collagen sections. The number of cells per high-powered magnification field (100X) was counted. 5 fields from each collagen section were examined to determine the mean number of cells recruited into the collagen implanted into naïve and vaccinated calves. n = 2 implants per group (10 fields counted per group). Data are presented as mean ± SD.

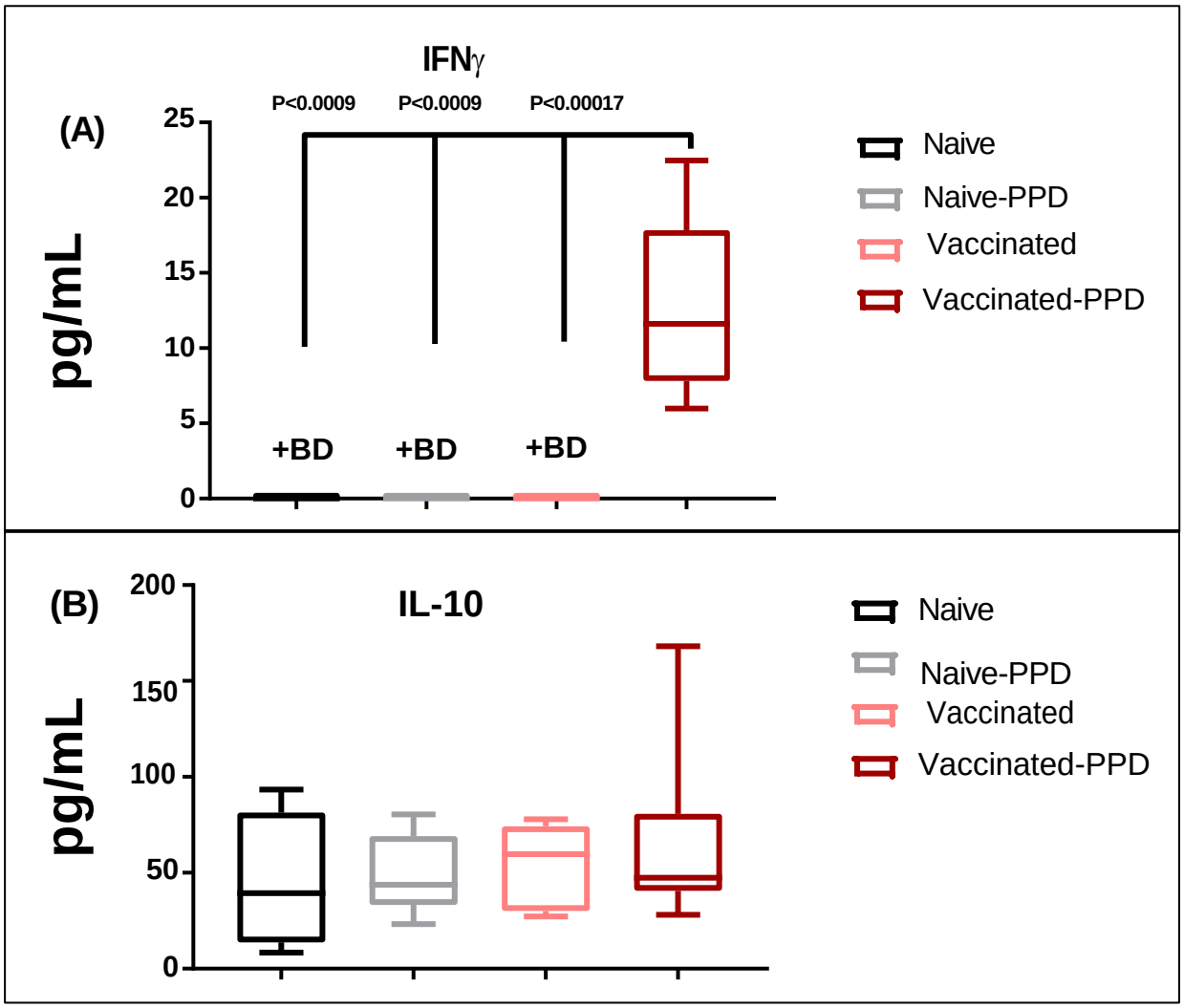

Figure 7: Cytokine levels from implant collagen. A – IFN-γ levels are highest in vaccinated calves where the collagen contains MAP antigen. B – IL-10 was detected in all implants. There were no differences in IL-10 concentrations in the collagen from different treatments. +BD: Below detection. Data are presented as mean ± SD.

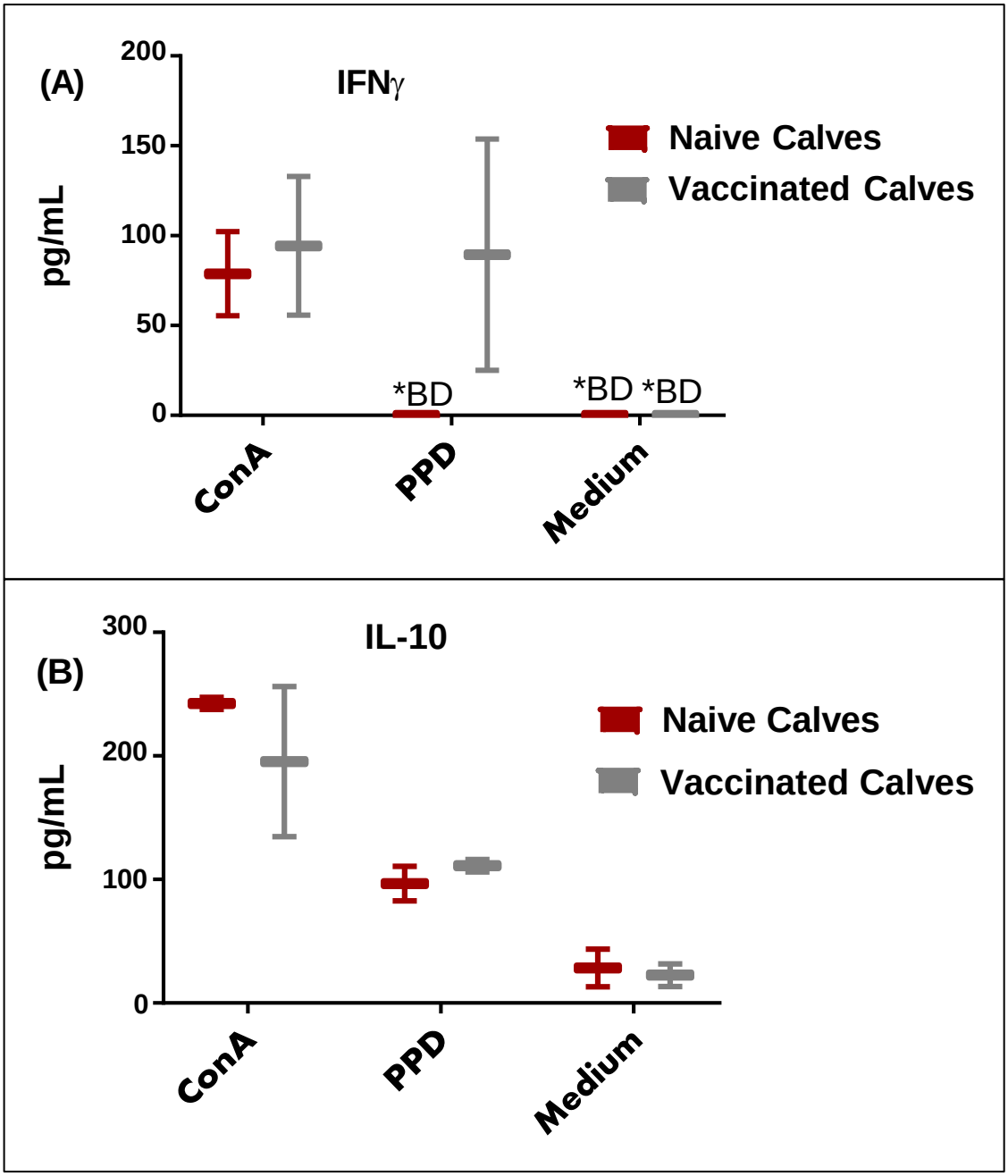

Figure 8: Cytokine levels in antigen stimulated peripheral blood mononuclear cells. A – IFN-γ is only detected in in stimulated PBMC from vaccinated calves. B – There are no differences in IL-10 in antigen stimulated PBMC from vaccinated or unvaccinated calves. ConA and medium were used as positive and negative controls (respectively). *BD: below detection. Due to experimental group size (n=2/group), statistical analysis could not be performed.

CHAPTER 3: LONGITUDINAL EVALUATION OF IN VIVO IMMUNE RESPONSES COLLECTED VIA SUBCUTANEOUS IMPLANT DEVICE RETRIEVED FROM MAP-VACCINATED AND NAÏVE CALVES

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Abstract

Johne's disease is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that results in significant economic losses for the cattle industry annually. Despite current limitations, vaccination holds future potential to effectively control and eliminate MAP infection. Therefore, the development of new vaccines with improved protection is currently an active area of research**.** One obstacle in the development of new vaccines is the difficulty in testing vaccine candidates. Bovine vaccine studies are difficult due to the long incubation period following infection causing these investigations to be lengthy and expensive. A long-term goal of our laboratory is to develop a simple, cost-effective method to specifically evaluate the host immune response to MAP to streamline the vaccine development process. The current study investigated the *in vivo*, cell-mediated immune (CMI) response to MAP antigen using a subcutaneous implant device designed previously by our laboratory. The eight-week, longitudinal study evaluated

the implant device's ability to collect immune responses *in vivo* in a population of MAP-immunized and naïve calves. The implants contained either collagen with saline only or collagen with MAP antigen, and cytokine (IFNγ and IL-10) levels within the implant were measured at 0, 2, 4, 6, and 8 weeks post-vaccination. IFNγ and IL-10 production by peripheral blood leukocytes (PBL) collected from the calves was also assessed at these time points. PBL isolated from vaccinated calves and stimulated with MAP antigen had significantly increased production of IFNγ over those isolated from naïve calves beginning 2 weeks post-vaccination and remained significantly increased throughout the study. No significant differences in IL-10 production by PBL were found. IFNγ levels in the MAP antigen-containing implants placed in vaccinated calves were significantly higher than the implants retrieved from naïve cattle at weeks 4 and 6 post-vaccination. These responses paralleled the responses observed in MAP-stimulated PBL from vaccinated calves. Significant differences between vaccinated and naïve calves in IL-10 levels within the implants were not observed. The intradermal caudal fold test (CFT) demonstrated false-positive rates when one of our calves reacted to PPD-J prior to vaccination. Therefore, our implant device was capable of identifying each of the 3 vaccinated calves, suggesting increased specificity over the intradermal CFT. Overall, the implant device consistently detects *in vivo* immune parameters and evidence shows this device may have a greater specificity than standard intradermal skin tests.

Introduction

Paratuberculosis is an enteric infection caused by *Mycobacterium avium subspecies paratuberculosis* (MAP) in cattle and other ruminant species [1]. Also known as Johne's

disease, paratuberculosis causes a chronic granulomatous enteritis leading to persistent diarrhea and loss of body condition [2]. MAP infection has an estimated presence on 68% of US dairy farms with economic losses to the industry estimated at $200-$250 million yearly [3, 4]. The greatest financial loss to the dairy industry is decreased milk production; however, early culling rates and poor reproductive outcomes also play a large role [5]. Currently, there is no treatment for MAP infected animals and mechanisms of preventative control, such as biosecurity measures, herd management, and vaccination, are emphasized to mitigate economic losses [1, 6]. Initial infection occurs in calves via the fecal-oral route during the early neonatal period, and vertical transmission from cow to calf is considered the primary route of infection [7]. Calves are most susceptible to MAP infection the first few days to months of life, and by one year of age, the risk for infection in adult cattle is low, indicating the host's immune response develops some degree of age-related MAP resistance [1, 7, 8]. It is known that only 10-15% of MAP-exposed calves will develop clinical disease and chronic wasting after a long incubation period that is known to last between 2-7 years [1, 2, 9]. This prolonged incubation period is known as Stage 1; in this period of infection, clinical signs are not observed and bacterial shedding is not detected. An obstacle facing MAP control within herds is the inability to detect Stage I animals before advancement into later disease stages (Stage II-III) in which cattle begin to shed bacteria in their feces [1, 10]. Therefore, vaccination is being sought for control and eradication of MAP due to the difficulty in identifying MAP-infected animals.

The first use of a MAP vaccine was described in 1926 using a live vaccine shown to decrease the severity of clinical disease [2]. Currently, three inactivated, whole-cell vaccine preparations (Mycopar®, Silirum®, and Guidiar®) are commercially available with

Mycopar® (Boehringer Ingelheim Vetmedica Inc.) being the only approved vaccine for MAP in the United States [11, 12]. Vaccination has been shown to be effective in decreasing fecal shedding and clinical disease [13]. Elimination of disease and economic benefit has been observed after 4-6 years of vaccination in previously infected, unvaccinated herds; this eradication of MAP infection was observed when vaccination was combined with other management and biosecurity techniques [14], Unfortunately, vaccination against MAP is not widespread to date due to some limitations of the current vaccines. Drawbacks to the currently available vaccines include: lack of complete protection from disease and fecal shedding, development of granulomas at the site of injection, and cross-reactivity with other mycobacterial species including *M. bovis* [11, 15].

Despite the current limitations, vaccination may still be the best way to effectively control or eliminate MAP infection in the future; development of new vaccines with greater protection is an active area of research. For instance, current research shows that subunit vaccines may have fewer drawbacks than whole-cell vaccines, such as the absence of granuloma formation at injection sites [16-18]. Research is also being conducted on live-attenuated strains. Live-attenuated strains contain various mutations that allow for a strong response from both the innate and adaptive immune systems without active infection, clinical disease, or pathogen transmission [19].

One obstacle in the development of new vaccines is the difficulty in testing vaccine candidates. Bovine vaccine studies are difficult due to the long incubation period following experimental infection which causes these investigations to be lengthy and expensive. As an alternative, cell culture and murine models have been used to study vaccine efficacy; however, these systems have not accurately predicted vaccine responses in the natural host

[8, 19]. An efficient and standardized natural host model is needed to quickly and effectively compare new vaccine candidates in vaccine trials across different laboratories.

Evaluation of the host immune response, in particular the detection of cell-mediated immune (CMI) parameters, has been a critical component in regard to testing vaccines candidates. Numerous investigations evaluating the post-vaccination immune response to MAP have used parameters associated with the T-helper 1 (Th1) and CMI response, such as IFNγ levels and intradermal skin thickness [20-22]. The focus of this research project is directed toward investigating the *in vivo*, CMI response of vaccinated and naïve cattle using a previously developed subcutaneous implant device. Prior work in our laboratory included the development of a self-contained, removable device capable of subcutaneous implantation and collection of immunological parameters. The purpose of the eight-week, longitudinal study reported here was to evaluate the implant device's ability to collect immune responses to MAP antigen *in vivo* in a population of MAP-immunized and naïve calves.

Materials and Methods

Animals and vaccination

Six Holstein bull calves were obtained by Laboratory Animal Resources at Iowa State University in Ames, Iowa. Calves weight approximately 250-300lbs and were housed in an outdoor research facility and divided randomly into the following two groups: naïve (n=3) and MAP vaccinated (n=3). Calves were allowed to acclimate to their environment for one week prior to vaccination and initiation of the study. The three calves receiving immunization were vaccinated subcutaneously in the ventral neck region with Mycopar®, an inactivated, whole-cell vaccine (Boehringer Ingelheim Vetmedica, Inc., Saint Joseph,

Missouri), per the manufacturer's instructions. Samples were collected from calves on day 0 and weeks 2, 4, 6, and 8 post-vaccination. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA, USA).

Implant device, collagen, MAP antigen

For each time point, 12 stainless steel implant devices (described in detail in Chapter 2) with 30um mesh inserts were autoclaved and loaded with *PureCol® EZ* Gel (Advanced BioMatrix, San Diego, CA). *PureCol® EZ* Gel is a commercially available, pre-formulated, purified Type I bovine collagen at 5mg/ml concentration that forms a firm gel at 37°C. The collagen contains CMEM/F-12 medium with L-glutamine and L-alanine-L-glutamine. *PureCol® EZ* Gel (hereafter referred to as collagen) was stored at 4°C in liquid form. A commercial preparation *Mycobacterium paratuberculosis* purified protein derivative johnin (PPD-J) intradermic tuberculin (NVSL, Ames, IA, USA) was used for this study and stored at 4°C. PPD-J is a solution of phenolized phosphate buffer and tuberculoprotein at a concentration of 1.0mg/ml. Prior to implantation, *PureCol® EZ* Gel containing 30% PPD-J or sterile saline, as a control in separate implants, was loaded into the mesh lining within the cylinder of the implant. Implants were then placed at 37°C for at least one hour allowing formation of a firm gel.

Implantation procedure

Using a halter and adjustable head gate, the six calves were restrained at day 0 for blood collection and implantation. The right and left lateral necks were sterilely prepped and

3ml of 10mg/ml lidocaine was dispersed into a localized area of approximately 4cm by 4cm. A 0.5cm incision was made via scalpel blade followed by insertion of the full implant using the implantation device. The implant was completely placed into the subcutis. A single interrupted suture was then placed to close the incision. Two implants were placed in each animal on opposite sides of the neck; the first, a control implant, containing collagen and saline and a second implant containing collagen and PPD-J. Ten milliliters of whole blood was collected in acid citrate dextrose via jugular venipuncture. After 48 hours, the calves were restrained for removal of the implant device. A single interrupted suture was placed in the skin to close each implant site after implant removal. The implantation, blood collection, and implant retrieval was repeated at 2, 4, 6, and 8 weeks. After transportation back to the laboratory, the nylon mesh and associated collagen plug were removed from the stainless-steel housing of each implant. The collagen was then removed from the nylon mesh lining. For histopathology, an approximately 1mm x 4mm section of collagen was placed in 10% neutral buffered formalin and processed routinely for hematoxylin and eosin (H&E) staining. For cytokine analysis, the remaining collagen was cooled on ice and minced with 100ul of complete RPMI media (cRPMI, Life Technologies, Grand Island, NY, USA). The supernatant was then collected and stored at -20°C for future cytokine analysis (n=3 implants per group, vaccinated and naïve, per time point).

Blood collection and cell culture

At each time point, six blood samples were collected from naïve (n=3) and MAP-vaccinated (n=3) calves. Twenty milliliters of whole blood was collected in EDTA via jugular venipuncture. Ten milliliters of the EDTA-treated whole blood was placed in a 50 milliliter

conical tube with 40 milliliters of ACK Lysing Buffer at room temperature for 5-10 minutes. Leukocytes were then collected by centrifugation at 1000g for 10 minutes. The cell pellet was washed twice with phosphate buffered saline (PBS) and cells were then collected by centrifugation at 1000 g for 8 minutes. The peripheral blood leukocytes (PBL) were then suspended in cRPMI to achieve a final concentration of $5x10^6$ cells/well. Cells were plated in a 96-well plate and stimulated with Concanavalin A (Sigma-Aldrich, St. Louis, MO, USA) at 2ug/ml, PPD-J (National Veterinary Services Laboratory, Ames, IA, USA) at 10ug/ml, or cRPMI medium for 24 hours at 37°C and 5% CO_2. The plate was spun at 300g for 5 minutes and supernatants were collected and placed at -20°C for future cytokine analysis.

Cytokine analysis

Supernatants from the implant collagen as well as the PPD-J and Concanavalin A-stimulated PBL were incubated with magnetic beads in a 96-well plate at room temperature for two hours. Magnetic beads (MC10062-01, MC10026-01, Bio-Rad Laboratories Inc., Hercules, CA, USA) were pre-conjugated with IL-10 and IFN-γ capture antibody (MCA2110, MCA2112, Bio-Rad). Biotin-labeled IL-10 and IFN-γ detection antibodies (MCA1783B, MCA2111B, Bio-Rad) were added and incubated for one hour followed by a 30 minute incubation with eBioscience™ Streptavidin PE (Invitrogen, Thermo Fisher Scientific Inc., USA). The mean fluorescence intensity and bead classification was determined via Bio-Plex®200 reader (Bio-Rad) and compared to a standard curve to derive cytokine concentrations of each individual sample. The following proteins were used to create a standard curve: recombinant bovine IFN-γ and recombinant bovine IL-10 (PBP007A, PBP016A, Bio-Rad). Samples were analyzed in two different batches, and

therefore calculated with two different standard curves; the 0 and 2 week time points were analyzed together and time points 4, 6, and 8 weeks were analyzed together. For analysis, IL-10 and IFNγ levels from PBL stimulated with media were subtracted from levels obtained from stimulated PBL from the same animal at the same time point.

Caudal fold test (CFT)

The caudal tail fold skin test was performed at the 0 week and 8 week time points as a tool to compare an established *in vivo* CMI response to the immune response of the implant in vaccinated calves. Calves were injected intradermally in the left (0 week) and right (8 week) tail fold with 0.1 ml of 1 mg/ml PPD-J and skin thickness is measured 72 hours post-injection. The skin thickness at week 0 and week 8 time points were measured via calipers; week 0 thickness was removed from week 8 thickness showing the overall change in thickness between vaccinated and naïve calves.

Graphs and data analysis

Graphs were made using GraphPad Prism 7 (La Jolla, CA). All data are shown as mean + standard error of the mean (SEM), and data sets were analyzed utilizing NCSS 12 Statistical Software (NCSS, LLC, Kaysville, Utah). Normality tests (D'Agostino-Pearson omnibus normality test) were performed on the datasets, and in some instances, the data did not have a normal distribution; therefore, nonparametric tests were used for all analyses. Cytokine levels were compared between vaccinated and naïve calves using Friedman test for repeated measures followed by Dunn's test for multiple comparisons. Skin test measurements were compared using Mann-Whitney test for unpaired data to compare ranks.

Results

Cytokine production in antigen stimulated PBL

Vaccination with Mycopar® showed significantly higher IFNγ production in PBL stimulated with the MAP antigen, PPD-J. IFNγ production by PBL isolated from vaccinated calves was elevated beginning 2 weeks post-vaccination compared with naïve controls and remained elevated throughout the 8 weeks of the investigation (Fig 1A). IFNγ levels peaked at week 4 post-vaccination, and gradually declined over the remainder of the study (Fig 1A). Stimulation of PBL with PPD-J resulted in greater IFNγ production than Con-A in vaccinated calves (data not shown). IL-10 levels were measurable at each time point throughout the study; however, significant concentration differences between naïve and vaccinated calves were not observed (Fig. 1B).

Cytokine production in implants

Levels of IFNγ within implants from calves vaccinated with Mycopar® were higher than those from naïve cattle beginning at week 2 post-vaccination. IFNγ was highest in vaccinated calves at the week 4 time point, and was significantly elevated over naïve controls ($P < 0.005$, Figure 2A). IFNγ levels in implants from vaccinated animals remained significantly ($P < 0.05$) elevated over naïve animals at week 6, before declining at week 8 (Figure 2A). There were no significant differences between vaccinated and naïve calves in IL-10 levels within the implants at any time point in the study (Figure 2B).

Temporal cytokine responses post-vaccination

Figure 3 compares the mean IFNγ concentration for PPD-J stimulated PBL and the implants containing PPD-J in calves vaccinated with Mycopar® over time. The *in vivo* pro-inflammatory cytokine response to MAP antigen paralleled the *in vitro* response, as evidenced by the similar temporal increase in IFNγ levels within the PPD-J-containing implants and those produced by PPD-J stimulated PBL collected from the same calves at the same time points. IFNγ production was maximal at week 4 post-vaccination for both PBL and implants; levels then declined gradually over the remainder of the study period. Naïve cattle had negligible IFNγ production by PBL in response to PPD-J and within the PPD-J containing implants (data not shown).

Caudal fold skin test

We used the PPD-J caudal fold test (CFT) to compare the implant response to a standard CMI test. Figure 4 shows the results before vaccination and at the 8 week time point. One calf exhibited increased thickness (10mm) at the CFT site before vaccination and thickness (10mm) in this calf at the 8 week time point remained the same. The remaining 2 calves demonstrated 75% and 81% increases in skin fold thickness after vaccination compared to the naïve group which had only a 7% average increase in skin fold thickness.

Discussion

MAP vaccination studies have generally shown the induction of a CMI response post vaccination that contributes to reduced fecal shedding and clinical disease in ruminants [12, 16, 17, 23-25]. Unfortunately, current vaccination strategies do not prevent infection or

transmission [11, 26, 27]. However, current subunit vaccine and attenuated vaccine candidates show potential as future immunizations that will protect against disease, eliminate fecal shedding, and prevent injection site granuloma formation [8, 16-18, 23, 28, 29]. To better evaluate these new vaccine candidates, a more efficient, predictive model for screening vaccine candidates is needed to assess protection in a natural host model. The rationale for this study was that measuring immune responses from within the animal will have benefit over *ex vivo* stimulated leukocytes and skin testing where cross reactivity with nonpathogenic mycobacteria is a current problem. The purpose of this longitudinal study was to expand on our laboratory's prior development of a self-contained, removable device capable of subcutaneous implantation and collection of immunological parameters. In this study, we were able to measure IFNγ within the implant collagen to identify which calves had received immunization. Our results showed the implants' ability to consistently and significantly detect MAP-specific IFNγ responses in vaccinated calves. These results are consistent with the IFNγ levels observed in human intradermal DTH site investigations. Chu et al. used immunohistochemical stains in DTH sites after BCG vaccination and found that 33% of cells (CD3+ T cells) at the DTH site contained IFNγ [30]. Previously, our lab detected an elevation in IFNγ when placing collagen and MAP into the subcutaneous tissues of calves compared to control sites consisting of collagen without MAP antigen [31].

This longitudinal study provided the opportunity to evaluate our implant's ability to measure cytokines at multiple time points post-vaccination. IFNγ levels at the 4 and 6 week time points were significantly greater in vaccinated compared to naïve calves. In contrast, the IL-10 measured within the collagen did not reveal any significant patterns to facilitate the identification of successful immune protection after MAP vaccination. Within peripheral

blood leukocytes, IL-10 demonstrated a small peak across all experimental groups at week 6 (45 days). This has been demonstrated in prior research by Stabel et al. in which IL-10 in MPS-simulated cultures was upregulated between 7 and 90 days in calves vaccinated against MAP. The significance of this IL-10 elevation is unknown; however, it may have implications in the development of the immune response of cattle in the period immediately following vaccination.

Figure 3 shows the correlation between the implants' IFNγ response and the response by the stimulated PBL. The graphs show that only the vaccinated calves stimulated with MAP antigen, either in cell culture or subcutaneously placed implants, exhibited detectable levels of IFNγ. The PPD-J stimulated leukocytes (red) and PPD-J containing implant (green) had similar patterns responses in vaccinated and naive cattle. This pattern of IFNγ response is similar to research investigating the CMI response at the known sites of MAP infection such as the ileum and lymph nodes. Coussens et al. revealed a greater expression of pro-inflammatory cytokines including IFNγ within the ileal tissues of infected cattle compared to naïve cattle. Systemic evaluation of cytokines is a well-researched topic in which cytokine profiles from stimulated PBMC cultures have demonstrated greater levels of IFNγ after immunization against and infection with MAP in ruminants [32-37]. Stabel et al. investigated the effects of immunization after vaccination with the killed, commercially, available vaccine, Mycopar® [22]. CMI parameters explored in this study included MAP-specific IFNγ levels in response to PPD-J as well as the intradermal comparative cervical skin test. Seven days after vaccination, strong IFNγ responses were measured and lasted until the study's completion at twelve months [22]. Similarly high concentrations of IFNγ were noted in other laboratories such as the long-term study of Mycopar®-vaccinated, Dutch dairy cows

by Muskens et al. In this study, high levels of IFNγ were observed throughout the two year period following vaccination in comparison to naïve, control cows [38].

The caudal fold test is a widely used diagnostic tool for detection of bovine tuberculosis and has shown good specificity when used as a diagnostic tool for MAP infection [2, 39, 40]. Monoghan et al. demonstrates the increase of IFNγ to correlate to an increase in skin thickness of the comparative skin test [21]. Stabel et al. also evaluated skin thickness after intradermal injection with PPD-*M. avium* and PPD-*M. bovis;* these results showed that immunized calves had a stronger reaction to PPD-*M. avium* compared to PPD-*M. bovis;* one calf was minimally and falsely reactive to PPD-*M. bovis* suggesting that 20% of the cattle in this study would be falsely diagnosed with MAP-infection [22]. We found similar false-positive rates in our vaccination study in which one of our calved reacted to PPD-J prior to vaccination. In our study, the skin thicknesses at week 0 and week 8 time points were measured. A significant difference between naïve and vaccinated cattle was not observed, likely due in part to one calf having a large thickness prior to and following vaccination. This is not an uncommon observation regarding intradermal mycobacterial testing due to cross-reactivity of environmental mycobacteria [22, 41-43]. The remaining 2 calves demonstrated 75% and 81% increases in skin fold thickness after vaccination compared to the naïve group which had only a 7% average increase in skin fold thickness. It is interesting that our implant was able to identify this calf as vaccinated, which may suggest increased specificity over the skin test.

Measuring cytokine expression profiles in PBLs and tissue samples provide beneficial information on mycobacterial immune responses and diagnostic markers at the systemic and gastrointestinal level. However, these evaluation methods are costly and require extensive

time in performing *in vitro* assays that may not indicate a protective response *in vivo.* Currently, minimal literature exists on the immune response at the level of the DTH site in cattle. This implant device demonstrated reproducible and significant measurements of IFNγ for detection of cell-mediated immunity and vaccination in cattle. This method for *in vivo* immune detection has the potential to provide a better understanding of the MAP vaccination-induced immune response by mimicking a

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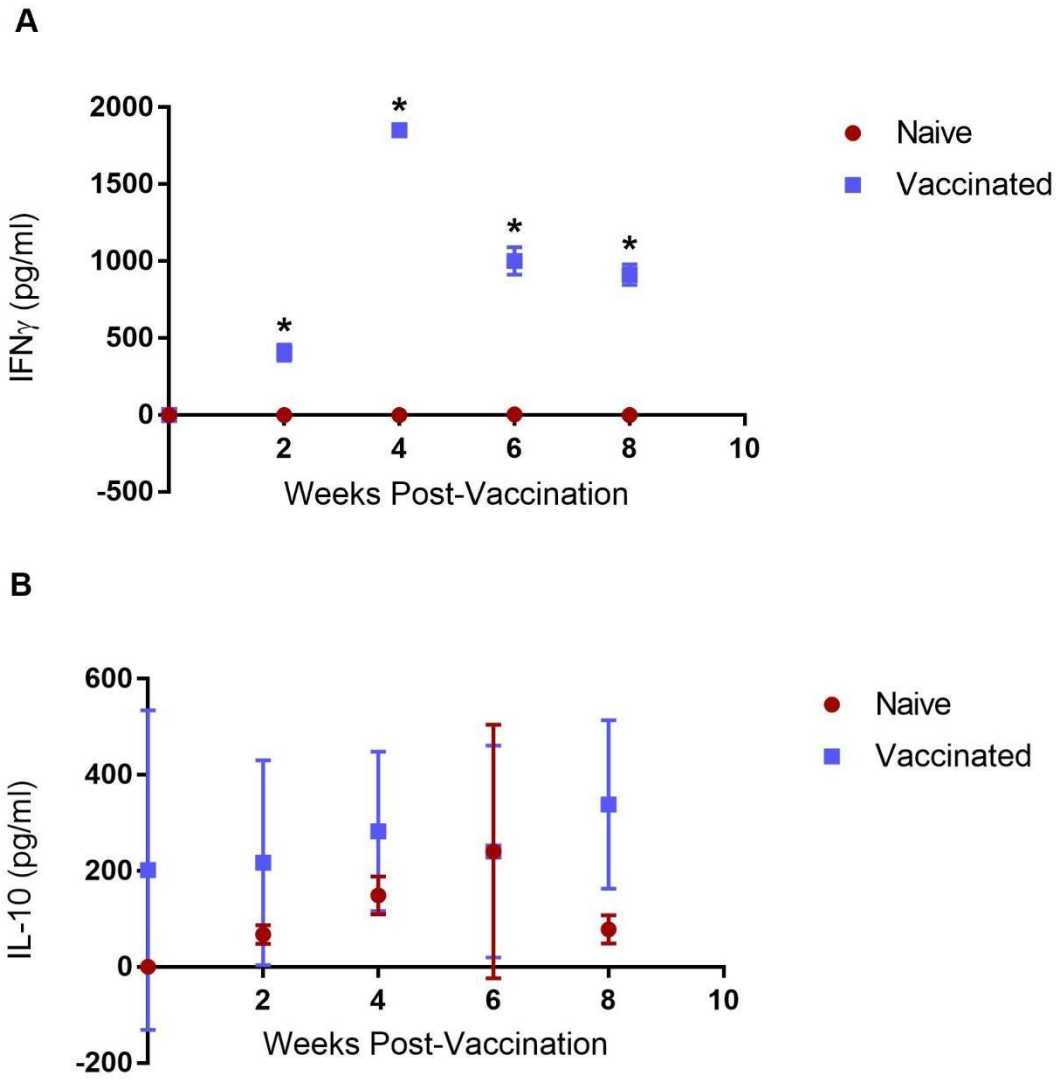

Figure 1. Leukocytes were collected from naïve and vaccinated calves at two-week intervals post-vaccination and were stimulated with MAP antigen, and cytokine production was measured via multiplex bead assay. A) IFNγ production secondary to stimulation with MAP antigen was significantly higher in vaccinated compared to naïve calves beginning at 2 weeks post-vaccination, and remained significantly higher throughout the study. B) IL-10 production following stimulation with MAP antigen was not significantly different between vaccinated and naïve calves (*, $P < 0.05$, Friedman test for repeated measures and Dunn's test for multiple comparisons).

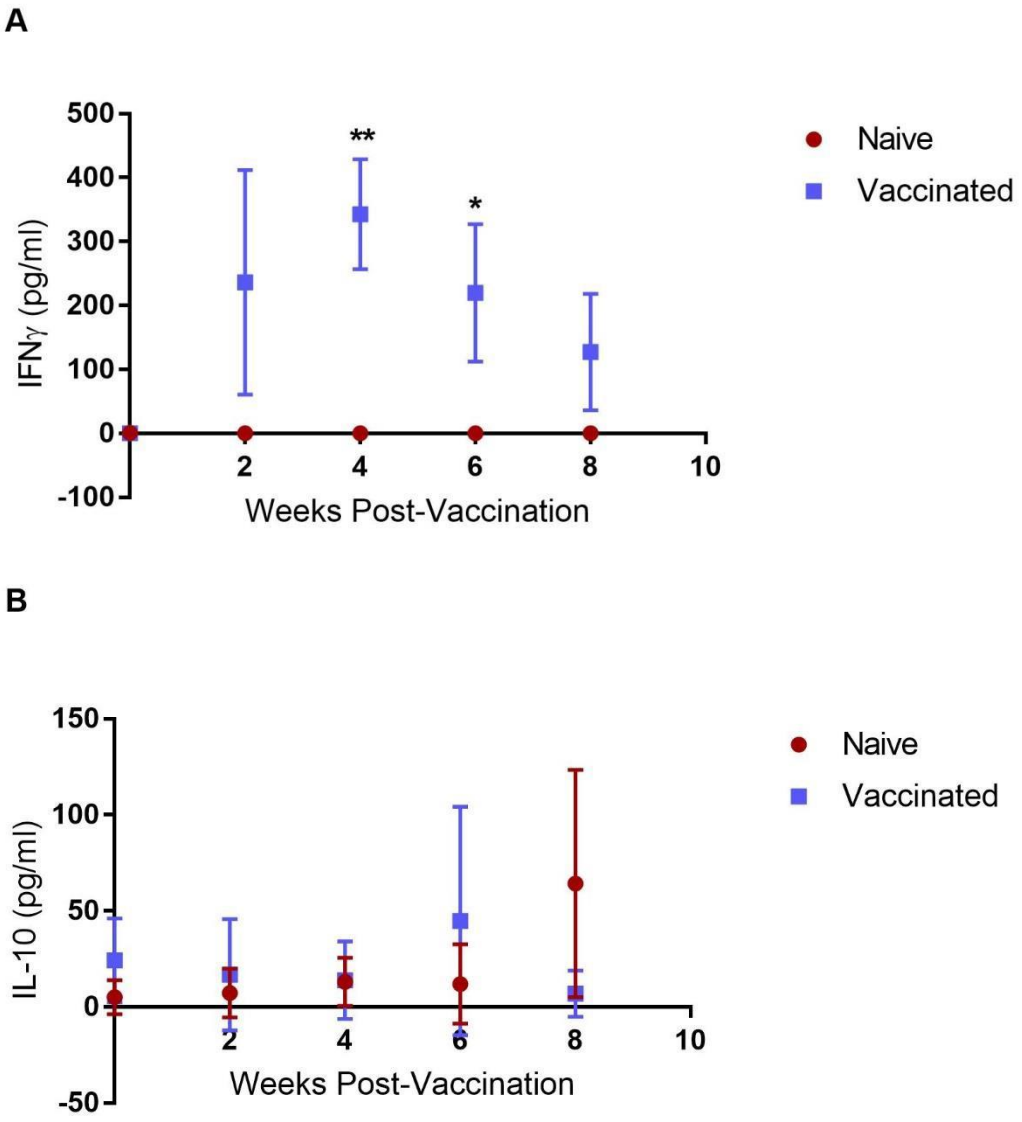

Figure 2. Implants containing collagen infused with MAP antigen (PPDJ) were placed subcutaneously in naïve and vaccinated calves for 48 hrs every two weeks following vaccination to capture immune responses. Cytokine levels within the implants were measured following removal via multiplex bead assay. A) IFNγ levels within the implants were significantly higher in vaccinated calves at 4 and 6 weeks post-vaccination. B) IL-10 levels within the implants were not significantly different between naïve and vaccinated calves at any time point (*, $P < 0.05$; **, $P < 0.005$, Friedman test for repeated measures and Dunn's test for multiple comparisons).

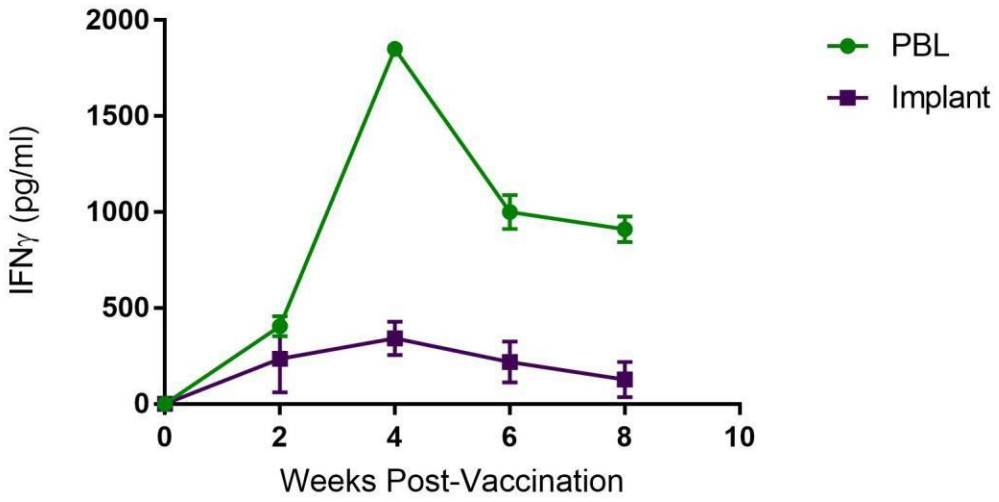

Figure 3. Comparison of IFNγ levels over time in calves vaccinated with Mycopar®. Temporal production of IFNγ in calves post-vaccination was assessed in both peripheral blood leukocytes (PBL) stimulated with MAP antigen and subcutaneous implants containing collagen infused with MAP antigen. IFNγ production by PBL paralleled the levels isolated from the implants *in vivo*, with the maximal response noted 4 weeks post-vaccination. Naïve cattle had negligible IFNγ production by PBL in response to MAP antigen and within the MAP-antigen containing implants (data not shown).

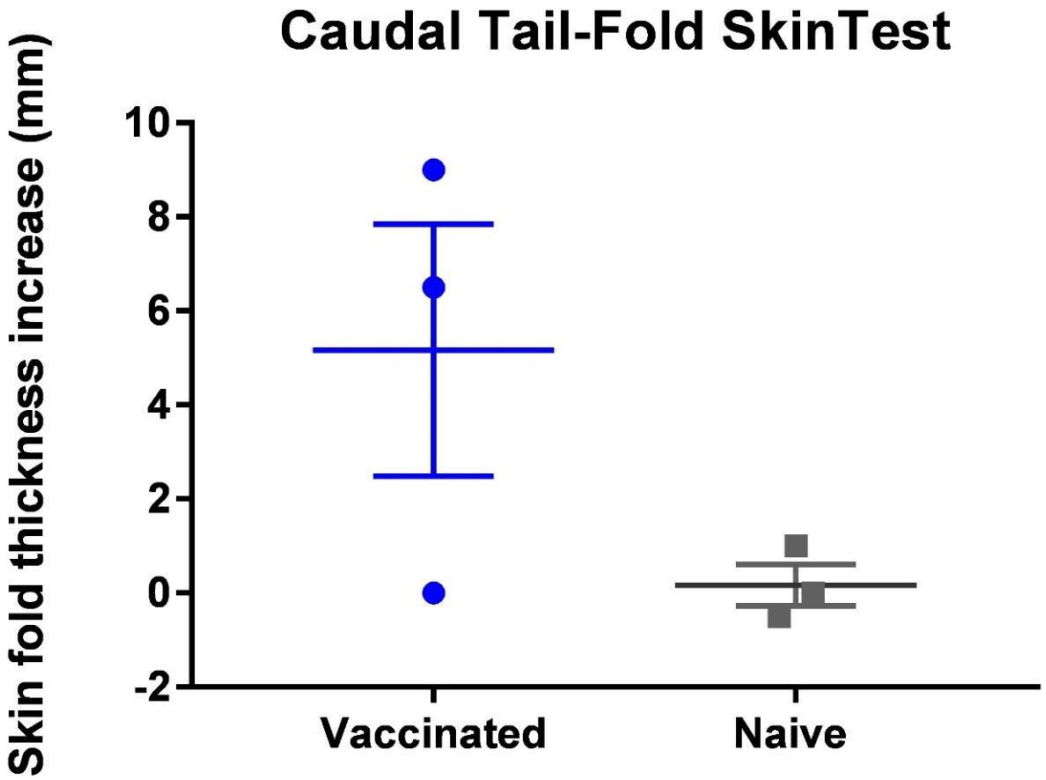

Figure 4. The PPD-J caudal fold test (CFT) was used as a standard established assessor of the cell-mediated immune response to Mycopar® vaccination. The increase in skin thickness at 8 weeks post-vaccination was recorded for each calf in the study. Calves that received the vaccine tended to have an increase in skin fold thickness compared to unvaccinated calves; however, one calf that received the vaccine had increased thickness at the vaccine site prior to vaccination.

CHAPTER 4. USE OF A SUBCUTANEOUS BACTERIAL CHALLENGE MODEL AS AN INTIAL SCREENING TEST FOR MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS VACCINE CANDIDATES

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic, infectious disease of cattle and other ruminants. MAP infection results in a slow and progressive enteric disease that leads to large economic losses in the dairy industry each year. Due the difficulty in identifying subclinical animals, vaccination programs may contain the greatest potential for control and elimination of MAP-infection in ruminants. The research in this project will build on a previously designed subcutaneous implant device capable of *in vivo* CMI collection of immune parameters; our investigation explored the device's potential as a bacterial killing assay. Collagen and MAP-containing implant devices (6 per animal) were placed in the subcutaneous tissues of calves divided into two groups, naïve (n=3) and Mycopar® vaccinated (n=3) at 14 and 20 days post-vaccination. After 5 days, implants were retrieved from the subcutaneous tissues and collagen was processed for evaluation of IFNγ cytokine levels and flow cytometric analysis of bacterial viability. Three weeks post-vaccination, flow cytometric analysis of the bacteria retrieved from collagen demonstrated a significantly greater percentage of propidium iodide (PI)

stained-MAP in the vaccinated calves compared to naïve calves, indicating enhanced bacteria killing within the vaccinated calves. Collagen from vaccinated calves at the three week time point also demonstrated a significantly higher production of IFNγ than collagen from naïve calves. By exploring the implant device's ability to assess bacterial viability with flow cytometry, this investigation demonstrated the potential for the device's use as a bacterial challenge model to efficiently and economically screen novel vaccine candidates in a natural host model.

Introduction

Paratuberculosis is a chronic, infectious disease of cattle and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP infection results in a slow and progressive enteric disease that leads to the clinical symptoms widely known as Johne's disease [1-3]. Due to a reduction of milk production and early culling of sick cattle, MAP infection is recognized as a major herd problem in the dairy cattle industry. Economic losses to the United States dairy industry are estimated between $200-250 million per year [4-6]. Various methods of prevention and control are currently emphasized such as improvement of biosecurity methods, test-and-cull management strategies, and vaccination programs [2, 7]. The largest obstacle to elimination of MAP infection is the difficulty in identifying MAP-infected cattle in the early stages of disease in which clinical symptoms are absent and MAP bacteria are shed into the environment [8, 9]. It is widely known that primary transmission occurs via fecal-oral transmission in the neonatal period; however, only a small percent of exposed cattle will develop chronic wasting disease after the 2-7 year latent period observed after transmission [2, 3, 9-11]. Due to this difficulty in identifying subclinical animals,

vaccination programs may contain the greatest potential for control and elimination of MAP-infection in ruminants.

Commercially available MAP vaccines are unable to provide full immune protection from MAP infection. These vaccines have been shown to reduce fecal shedding and delay clinical disease progression; however, vaccination does not prevent fecal shedding or disease transmission [12, 13]. Although vaccine strain development is an active area of research in the MAP world, a crucial need exists for a new method to efficiently and economically screen novel vaccine candidates. One of the largest obstacles in the development of new vaccines is the poor correlation between initial screening tests and secondary evaluation methods in the sheep and cattle natural host models. For instance, cell culture models and murine models have been used as initial vaccine screening tests, but do not reliably predict the vaccine response in a natural host model [10, 14, 15]. Previous investigations in our laboratory included work toward the development of an initial vaccine screening test for use in the natural bovine host model. In prior studies we used a self-contained, removable device capable of collecting immune parameters and measuring *in vivo* cell-mediated immunity (CMI) in the subcutaneous tissue of cattle. The implant device was able to successfully identify vaccinated vs naïve calves between 2 and 8 weeks post-vaccination. The research proposed here will use our subcutaneous implant as a platform for a bacterial killing assay in vaccinated calves.

A wide variety of bacterial challenge models and assays are currently used in paratuberculosis research. Post-vaccination challenge models explore the response of the immune system and its ability to eliminate pathogens and avoid disease. For instance, common assessment methods, due to their cost-effective nature, are *in vitro* assays such as

survival rates in infected bovine cultured macrophages [16, 17]. Kabara et al. performed a macrophage culture study to demonstrate that populations of MAP-infected macrophages contain fewer apoptotic cells than control cells, and that MAP infection reduces the sensitivity of infected macrophages to induction of apoptosis by H_2O_2 [16]. In addition, various oral and inoculation challenge models exist in numerous species including mice, sheep, goats, and cattle. Unfortunately, this lack of a standardized challenge model makes comparison of new vaccine candidates across different laboratories highly challenging [18]. However, recent advancements toward standardizing animal challenge models and vaccine trials for MAP have been implemented. Supported by the Johne's Disease Integrated Project (JDIP), a committee of MAP researchers was formed called the JDPI Animal Model Standardization Committee (AMSC). Committee members formulated guidelines and specific parameters for each MAP host model [19]. In a paper by Bannantine et al. evaluating 22 vaccine candidates, a 3-phase investigation was implemented using the standardized testing parameters [10, 14]. Phase 1 evaluated survival in bovine macrophages and Phase II used the murine model to evaluate the bacterial load in tissues at varying time points after MAP challenge. Finally, Phase III was conducted in goat kids [14, 18]. The results of the study indicated that the use of the 3-phase, macrophage to mouse to ruminant platform may need adjustments, particularly in the macrophage and mouse models where evidence of attenuation *in vitro* and within an unnatural host, respectively, does not appear to predict natural host protection [14, 17, 18].

Another post-vaccination challenge model is the use of *in vitro* and *in vivo* bacterial killing assays to examine the influence of vaccination on the immune system's ability to kill MAP organisms. Post-vaccination screening assays to detect bacterial death or clearance

serve as biomarkers for immunocompetence to immunization. A wide variety of bacterial viability techniques are used in research including culture based techniques, cell integrity assessments, sequencing approaches, measurements of RNA, DNA, RT-PCR and qPCR , ATP detection, isotype probing, and membrane integrity assessment with selective stains [20]. For this experiment, staining for membrane integrity was chosen to assess MAP viability within the retrieved collagen implants. Propidium iodide (PI) is a red-fluorescent dye that permeates compromised cell membranes and binds to internal nucleic acids. PI staining is a widely used technique for rapid enumeration of dead bacteria. Lahiri et al. has demonstrated the success of PI in assessing the membrane integrity of *Mycobacterium leprae* in a murine study [21]. A second study by Hendon-Dunn et al. used flow cytometry to visualize fluorescently labeled bacteria [22]. The use of flow cytometry to assess PI stained bacteria allows researchers to gate and enumerate the number of dead bacteria. [22-25]. The research in this project will build on a previously designed subcutaneous, *in vivo* CMI collection device; our investigation will explore the device's potential as a bacterial killing assay. A subcutaneous bacterial challenge model could prove to be an efficient and cost-effective method for screening novel vaccine candidates in a natural host model.

Materials and Methods

Animals and vaccination

Four to six-week-old castrated Holstein bull calves were obtained by Laboratory Animal Resources at Iowa State University in Ames, Iowa and housed in an outdoor research facility for two weeks. Calves were divided randomly into two groups, naïve (n=3) and vaccinated (n=3) and allowed to acclimate for three days prior to vaccination. Three calves

received immunization and were vaccinated subcutaneously in the ventral neck region with Mycopar®, an inactivated, whole-cell vaccine preparation made by Boehringer Ingelheim Vetmedica, Inc., Saint Joseph, Missouri), per the manufacturer's instructions. Fourteen days post-vaccination, calves were moved to isolation housing at the Iowa State University College of Veterinary Medicine Livestock Infectious Disease Isolation Facility (Ames, IA). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA).

Bacterial inoculum

The MAP strain K10 was obtained from Dr. John Bannatine with the United States Department of Agriculture (USDA) Agriculture Research Service (ARS) in Ames, Iowa. The bacterial culture was maintained in Middlebrook 7H9 broth supplemented with mycobactin J. Bacterial concentration was determined by measuring absorbance at 540nm in comparison to a standard curve of MAP optical density at 540nm. Bacteria were then adjusted to a final concentration of $3.3 \text{ X } 10^7$ CFU/mL and suspended in our collagen, Matrigel®. The MAP K10 used in this project demonstrated greater than 90% viability prior to resuspension into Matrigel® collagen.

Implant, collagen, and experimental procedure

At two time points post-vaccination, 14 and 20 days, calves were implanted with collagen and MAP-containing steel implants. Thirty-six implant devices (described in detail in Chapter 2) were autoclaved and loaded with Matrigel® Matrix (Corning Life Sciences, Tweksbury, MA) and MAP. Matrigel® Matrix is a commercially available, solubilized

basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor with multiple extracellular matrix proteins, including laminin, collagen IV, heparin sulfate proteoglycans, entactin/nidogen, and a number of growth factors. Matrigel® Matrix has a protein concentration of 8-12 mg/ml and forms a firm gel at room temperature. Prior to implantation on day 14 post-vaccination, Matrigel® Matrix containing $1 \text{ X } 10^7$ CFU was loaded into the mesh lining within the cylinder of each implant. The bacterial load was increased for the second set of implants on day 20 post-vaccination; Matrigel® Matrix contained $1 \text{ X } 10^8$ CFU per implant for this second group of implants. Implants were then placed at 37°C for at least one hour. On day 14 post-vaccination, six calves were restrained for blood collection and implantation using a halter and adjustable head gate. The right and left lateral necks were sterilely prepped and 3ml of 10mg/ml lidocaine was dispersed into three localized areas on each side of the neck. A total of six implants (three implants per side) were placed in each calf. Approximately 0.5 cm incisions were made via scalpel blade followed by insertion of the full implants using the implantation device. Single interrupted sutures were placed to close the incisions. Ten milliliters of whole blood were collected in EDTA via jugular venipuncture. After 5 days, the calves were restrained for removal of the implant device, and a single interrupted suture was placed in the skin to close each implant site. The implantation and blood collection were repeated at day 20 post-vaccination. At day 25 post-vaccination, calves were humanely euthanized for implant retrieval, collection of tissue surrounding implant, and collection of regional draining lymph node.

After transportation back to the laboratory, the nylon mesh and associated collagen plug were removed from the stainless-steel housing of each implant. The collagen was then removed from the nylon mesh lining. Implants were halved for various future analyses

including: histopathology, cytokine analysis, and flow cytometry. In total, six implants were removed per calf and halved for various analyses. Per calf, three halves were formalin fixed for histopathologic examination, three halves were shared with collaborators for future RNA isolation and genetic sequencing, three halves were used for flow cytometric analysis and viability, and the remaining three halves were used for collection of cytokines (round 2 implants) and duplicates for flow cytometric analysis (round 1 implants).

For histopathology (n=9 per group per time point), a section of each implant, subcutaneous tissue surrounding implant, and regional lymph node tissue were placed in 10% neutral buffered formalin and processed routinely for hematoxylin and eosin (H&E) and Ziehl–Neelsen acid-fast staining. Immunohistochemical staining with the macrophage-specific calcium-binding protein, Iba-1, was used to assess for macrophagic infiltrate into the collagen sections. A granulomatous enteritis was used as a positive control. An antigen retrieval step using 10mM sodium citrate buffer of pH 6.0 was performed. Slides were then washed in 3% hydrogen peroxide and blocked with 3% bovine serum albumin and 0.05% Triton-X in tris-buffered saline. Four incubations followed: Iba-1 (1:500 dilution, 16 hours)(Abcam Inc., Cambridge, MA); ImmPRESS Horseradish Peroxidase (HRP) Polymer Reagent (per instructions, 30 minutes)(Vector Laboratories, Burlingame, CA); VECTOR NovaRED Peroxidase (HRP) Substrate Kit (per instructions, 5 minutes) (Vector Laboratories); Hematoxylin Gill Sol. No 2 (1.5 minutes) (Sigma-Aldrich, St. Louis, MO).

For cytokine analysis, a halved-implant section was cooled on ice and minced with 100ul of complete RPMI media (cRPMI, Life Technologies, Grand Island, NY, USA). The supernatants were then collected and stored at -20°C for future cytokine analysis (n=9 per

group, vaccinated and naïve, day 20 post-vaccination). For bacterial viability analysis, a section of collagen was processed (described in section 2.7) for flow cytometric analysis.

Blood collection and cell culture

Blood samples were collected from naïve (n=3) and MAP vaccinated (n=3) calves five days after implantation at each time point. Ten milliliters of whole blood was collected in EDTA via jugular venipuncture and placed in a 50 milliliter conical tube with 40 milliliters of ammonium-chloride-potassium (ACK) Lysing Buffer at room temperature for 5-10 minutes. Leukocytes were then collected by centrifugation at 1000g for 10 minutes. The cell pellet was washed twice with phosphate buffered saline (PBS) and cells were then collected by centrifugation at 1000g for 8 minutes. The leukocytes were then suspended in cRPMI to achieve a final concentration of $5x10^6$ cells/well after gating out cells less than 6um and greater than 12um in diameter. Cells were plated in a 96-well plate and stimulated with Concanavalin A (Sigma-Aldrich, St. Louis, MO, USA) at 2ug/ml, purified protein derivative-johnin (PPD-J, National Veterinary Services Laboratory, Ames, IA, USA) at 10ug/ml, or cRPMI medium for 24 hours at 37°C and 5% CO_2. The plate was spun at 300g for 5 minutes and supernatants were collected and placed at -20°C for future cytokine analysis.

Cytokine analysis

Supernatants from the implant collagen as well as the PPD-J and Concanavalin A-stimulated peripheral blood leukocytes (PBL) were incubated with magnetic beads in a 96-well plate at room temperature for two hours. Magnetic beads (MC10062-01, MC10026-01, Bio-Rad Laboratories Inc., Hercules, CA, USA) were pre-conjugated with IL-10 and IFN-γ

capture antibody (MCA2110, MCA2112, Bio-Rad). Biotin-labeled IL-10 and IFN-γ detection antibodies (MCA1783B, MCA2111B, Bio-Rad) were added and incubated for one hour followed by a 30-minute incubation with eBioscience™ Streptavidin PE (Invitrogen, Thermo Fisher Scientific Inc., USA). The mean fluorescence intensity and bead classification was determined via Bio-Plex®200 reader (Bio-Rad) and compared to a standard curve to derive cytokine concentrations of each individual sample. The following proteins were used to create a standard curve: recombinant bovine IFN-γ and recombinant bovine IL-10 (PBP007A, PBP016A, Bio-Rad).

Flow cytometry for viability

Flow cytometry was performed to assess bacterial viability on the retrieved collagen. A section of implant collagen was placed in 0.5ml radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific Inc., USA) for 10 minutes on ice. Samples were then vortexed for 30 seconds and any remaining large particles were removed using a 40 um cell strainer followed by 10ml of phosphate-buffered saline. Samples were centrifuged for 20 min at 3000 rpm, and the pellet was suspended in 300ul of 0.9% saline. Cells were then incubated at room temperature for 20 minutes with 0.2 ug/ml fluorescein diacetate (FDA). Propidium iodide (PI) was added to the samples at a concentration of 2 ug/ml and samples were run using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with FlowJo V10 software (Tree Star, Inc. San Carlos, CA).

Caudal tail-fold skin test

The caudal tail fold skin test was performed at the end of the study. Calves were injected intradermally in the right tail fold with 0.1 ml of 1 mg/ml PPD-J and skin thickness was measured 72 hours post-injection.

Graphs and analysis

Graphs were made using GraphPad Prism 7 (La Jolla, CA). All data are shown as mean +/- standard error of the mean (SEM). Normality tests (D'Agostino-Pearson omnibus normality test or Shapiro-Wilk normality test) were performed on the datasets, and in some instances, the data did not have a normal distribution; therefore, nonparametric tests were used for some analyses. Differences between MAP viability (Fig. 1, 2), IFNγ levels (Fig. 3), and skin test thickness (Figure 5) from retrieved collagen was assessed using a non-parametric Mann-Whitney test. IFNγ levels measured from stimulated peripheral blood leukocytes (Fig. 4A, B) were assessed using a Kruskal-Wallis test for multiple comparisons.

Results

Flow cytometric analysis of bacterial viability

Immunologic responses to vaccination were characterized by flow cytometric analyses of bacterial viability. Figure 1 and 2, A-D show how the gate for dead bacteria (PI+) was established and Figure 1 and 2, E-G demonstrate the differences, if observed, between the vaccinated and naïve group. Although FDA staining was performed, interference with the Matrigel® caused a spill-over signal into the PI+ channel; therefore, an accurate FDA+ bacterial population could not be determined and comparison of bacterial viability was

assessed between naïve and vaccinated groups by total PI+ bacteria. Fourteen days post-vaccination (Fig. 1), the collagen implanted for 5 days (implanted on day 14, retrieved on day 19) did not demonstrate significant differences in PI+ MAP between naïve and vaccinated calves. The second group of implants (implanted day 20 and removed on day 25) shown in Figure 2 showed that the collagen retrieved from calves in the third week after vaccination demonstrated a significantly greater percentage of PI+ (dead) bacteria. In this second group of implants, the initial number of live bacteria was higher at $1x10^8$ CFU/ml.

IFNγ from implants and stimulated peripheral blood leukocytes

Vaccination with Mycopar® resulted in significantly higher ($P = 0.0022$) IFNγ production from the collagen retrieved from vaccinated calves during the third week (day 20-25) post-vaccination (Fig. 3). Cytokine levels were not assessed at the 2 week time point as the collagen was used for other analyses. IFNγ levels were measured in the supernatants of stimulated PBL (Fig. 4); concentrations of IFNγ from cells cultured in medium alone were subtracted from cells stimulated with the positive control, ConA, and PPD-J before analysis. Statistically significant differences were not observed between the ConA and PPD-J stimulated leukocytes of vaccinated and naïve calves. While trends were present for IFNγ between vaccinates and naïve calves, statistical significance was not achieved.

Caudal tail-fold test

As a second measure of cell-mediated immunity, the caudal tail-fold test with PPD-J was performed and skin thickness between the vaccinated and naïve groups was assessed (Fig 5, Table 1). Statistical differences were not observed between vaccinated and naïve

cattle; however, an overall trend was observed in which the vaccinated calves had skin thickness measurements ≥ 10mm compared to the naïve group with measurements of ≤ 5mm.

Histopathology

All implant sections were processed and stained with H&E and Ziehl-Neelsen acid-fast staining. Figure 6A, B show H&E sections at 10x with 40x insets; overall, a trend toward higher cellularity within the collagen retrieved from vaccinated calves was observed during the second week post vaccination. A trend in immune cell infiltrate cellularity was not observed in the sections from the implants collected from the third week post-vaccination. In all groups, the majority of cells are degenerate neutrophils with lesser numbers of macrophages and lymphocytes Figure 6C, D shows acid-fast staining of collagen sections from naïve and vaccinated calves. A trend in the amount of bacteria was not observed, and MAP was observed intracellularly within both groups of calves. Immunohistochemical staining with the macrophage-specific calcium-binding protein, Iba-1, was used to assess for macrophagic infiltrate into the collagen sections and a positive control is observed in Figure 6E. Rare Iba-1 positive cells were found in the collagen of vaccinated calves (Fig. 6F), but not noted in the collagen sections retrieved from naïve calves (Fig. 6G).

At the study's completion, the tissue surrounding an implant as well as a prescapular lymph node was collected in each calf for histological analysis. Figure 7A-C show increasing magnification of the H &E stained subcutaneous tissue surrounding the previously placed implant. Red asterisks illustrate the space that was occupied by the implant. The H&E stains reveal a marked inflammatory response comprised of numerous macrophages and degenerate neutrophils with low numbers of lymphocytes. This inflammatory response is greatest in the

area directly surrounding the implant; inflammation appears to decrease with distance from the implant. A Ziehl-Neelsen acid-fast stain was performed on the subcutaneous tissue and Figure 7D, E show intracellular and extracellular bacteria within the tissues surrounding the implant. Overall, low numbers of MAP were observed in the subcutaneous tissues of some calves and appeared independent of vaccination status. Finally, Figure 8 shows the 40x magnification of acid-fast stained prescapular lymph node sections in naïve (Fig. 8A) and vaccinated (Fig. 8B) calves. Low numbers of MAP were observed in the prescapular lymph nodes of some calves and appeared independent of vaccination status.

Discussion

Vaccination programs used in the dairy cattle industry are primarily designed to prevent diseases that result in production losses and early culling [12]. Several existing bovine vaccines, including the available MAP vaccines, have been successful for controlling or reducing many infectious diseases in herds [15, 26, 27]. Advantages to MAP vaccination include reduction in numbers of clinical stage animals and decreased fecal shedding of bacteria [15, 28-31]. It is well-known that commercially available vaccines do not eliminate fecal shedding, and therefore do not eradicate disease transmission in a dairy herd [10, 15, 28-30]. Development of new vaccines against paratuberculosis is an area of active research with protein subunit, DNA, and attenuated-live vaccines being investigated as potential candidates [10, 12, 15]. The ideal vaccine for MAP infection would induce a sustained cell-mediated immune response that prevents bacterial shedding and disease progression [10]. In addition, an ideal vaccine candidate would not form an injection site granuloma that is currently observed following MAP vaccination [32-34]. A new vaccine with improved

protection could provide control and elimination of MAP infection in dairy herds; however, current vaccine research is slowed due to the extensive costs and lengthy time frame necessary for vaccine testing. The development of a rapid and economical vaccine screening method is needed to efficiently test novel vaccine candidates in natural host models.

The primary goal of this study was to investigate a subcutaneous bacterial challenge model for use as an initial screening method of novel MAP vaccine candidates. The rationale behind this method is based on the inability of current screening methods, such as murine models and cell culture systems, to accurately predict the protective immunity in natural host models such as cattle, sheep, goats, and deer [10]. Use of a rapid and simple bacterial challenge model could substantially save time and costs involved in testing a vaccine strain that showed promise in a cell culture model but is not protective in the natural host.

For this study's purpose we chose a bovine host model and used the commercially available vaccine, Myopar®, to test our device's ability to function as a bacterial killing assay. We demonstrated that vaccination with Mycopar® vaccine elicited a strong IFNγ response to MAP within the collagen when compared to naïve cattle. These findings are consistent with the findings observed in Chapters 2 & 3 in which vaccinated cattle consistently demonstrated an IFNγ response to the MAP antigen, PPD-J, compared to naïve calves. This suggests that the implants elicit reliable cytokine responses to MAP organism and MAP antigen. These results are also consistent with numerous studies detecting an increase in IFNγ and other Th1, pro-inflammatory cytokines within ileal and lymph node tissues of MAP infected cattle [35-38]. For instance, Lee et al. used immunohistochemistry and in situ hybridization techniques to demonstrate a similar increase of IFNγ expression in the ileal tissues of clinically infected cattle that was not observed in nonclinical animals [37].

It should also be noted that when compared to other standard methods of CMI detection, such as DTH caudal tail-fold skin testing and peripheral blood stimulation, the IFNγ response detected within the collagen was the only method to consistently find significant differences between vaccinated and naïve groups of cattle. In our study, differences were not observed between groups when comparing skin test thickness or IFNγ levels in peripheral blood stimulation. These findings are similar to the findings we observed in Chapter 2 in which one of the calves exhibited a strong response to the intradermal DTH test prior to MAP vaccination. Similar studies have been performed in which poor specificity of DTH tests were observed in vaccinated herds. Hines et al. evaluated the specificity of the johnin DTH skin test in MAP-vaccinated goats. The study used a caprine model to look at various immune parameters following vaccination with 5 experimental, attenuated strains of MAP vaccine candidates compared to the commercial control vaccine, Silirum®. Intradermal skin testing was performed at various time points before and after vaccination using three types of PPD: PPD-*M. avium*, PPD-*M.bovis*, and PPD-johnin. The results from this study showed that the commercial vaccine, Silirum®, as well as some of the experimental vaccines, produced false-positive results with the all three PPD skin tests [39]. The low specificity observed with intradermal DTH testing is likely due to cross-reactions or previous sensitization of animals to environmental mycobacterial species. Many antigens are shared between mycobacteria, and non-specific responses are not uncommon observations [40].

Flow cytometry using propidium iodide (PI) staining was used to enumerate percentage of dead bacteria from total bacteria. The host response to the bacteria-containing collagen revealed no significant differences in vaccinated and naïve cattle at the two week post-vaccination time point. Percentages of dead bacteria at this time point were low and

ranged between 2-9% of total bacteria within the implant. However, flow cytometric analysis of bacterial viability at three weeks post-vaccination did comprise a significantly greater percentage of PI+ bacteria with an average percentage of dead bacteria of 29.5% and 44.6% in naïve and vaccinated cattle, respectively. These results indicate that vaccinated calves can be screened for vaccine-induced killing responses as early as 3 weeks post-vaccination. This finding parallels a longitudinal study by Stabel et al. that followed the host immune response for 1-year post-vaccination. In this study, IFNγ secretion in PPD-J stimulated PBMCs were measured in vaccinated and control calves at day 0,7,14,30, 90, 180, 270, and 360; Stabel et al. showed a steady increase in IFNγ levels after vaccination with a peak level of IFNγ at 30 days post-vaccination. Stabel et al. also demonstrated a drop in IL-4 concentration secreted from stimulated PBMCs between 14 and 30 days post-vaccination. An increase in antibody was also observed after 14 days on an immunoblot of sera. These vaccine-induced immunological findings by Stabel et al. regarding stimulated PBMC IFNγ and IL-4 levels as well as antibody acquisition all occurred between day 14-30 post-vaccination; similarly, in the implant collagen described in Chapter 2, a peak in IFNγ concentration was observed at 4 weeks post-infection. It is hypothesized that the switch from low percentages of dead bacteria at 2 weeks post-vaccination to the greater levels observed at 3 weeks, may be correlated to these alterations in immune parameters observed between 14-30 days post-vaccination [26]. Furthermore, the first round of implants contained $1x10^7$ CFU/implant compared to the second round of implants that contained $1x10^8$ CFU/implants; it is possible the low bacterial load in the first round of implants was not large enough to mount a significant Th1 response in only 5 days. Future studies may benefit from an increased duration of implantation.

The collagen implants retrieved from both calf groups contained an immune cell

infiltrate comprised morphologically of a large population of markedly degenerate neutrophils. Low numbers of macrophages and lymphocytes were noted. The first round of implants at 2 weeks post-vaccination exhibited a greater cellular density in vaccinated calves than observed in the collagen of naïve calves; however, this trend was not observed in the histological sections from the implants at 3 weeks post-vaccination. It is speculated that the loss of differences in cell density between the groups is attributed to the rapid re-implantation of the second group of implants only 24 hours after retrieval of the first implants; when the second round of implants were placed, the collagen-containing device was positioned into an environment with an immune response primed by the first round of implants. This priming of the tissues prior to implantation may have allowed a similar degree of cellular infiltrate, particularly neutrophils, to migrate through the collagen regardless of vaccination status. With acid-fast staining, a trend in bacterial load was not observed between the different experimental groups; however, presence of intracellular bacteria was observed in all implants. The detection of intracellular bacteria in the collagen implant demonstrated that the device's ability to attract leukocytes for migration into the collagen and subsequent bacterial phagocytosis.

Immunohistochemical staining with the macrophage-specific calcium-binding protein, Iba-1, was used to assess for macrophagic infiltrate in the collagen sections, and only rare Iba-1 positive cells were found in the collagen of vaccinated calves. However, histology of the tissues surrounding the implant revealed a marked inflammatory response with a large number of macrophages present. Acid-fast staining revealed the presence of bacteria in the surrounding tissues of some calves and the prescapular lymph node of one calf; quantity of acid-fast bacterial load appeared independent of vaccination status. The significance of the

rare macrophages within the implant from a vaccinated calf is unknown. One theory is that the degree of macrophagic inflammation outside the implant needed more time to migrate into the collagen, and repeat studies of extended duration may be beneficial. Although macrophages were only noted in one implant, a second theory is the ability of the vaccinated calf to attract macrophages into the collagen for bacterial engulfment; it is well-known that a previously primed Th1 cell infiltrates the site of exposure and recognizes peptide:MHC II complexes. This is followed by secretion of pro-inflammatory cytokines, such as IFNγ, leading to the activation and recruitment of more macrophages to the exposure site [41].

In conclusion, we adapted a previously designed subcutaneous implant device and explored its ability to function as a bacterial killing assay. Through fluorescently labeled bacteria and flow cytometric analysis, we were able to enumerate the dead bacteria in naïve and vaccinated calves. Three weeks post-vaccination, a significantly higher percentage of dead bacteria within the implant collagen was observed; this finding suggested that vaccinated calves can be evaluated for vaccine-induced killing responses and serve as a potential screening test; this screening test would facilitate selection of vaccine candidates for more extensive, Phase III, vaccine trials. A subcutaneous bacterial challenge model could prove to be an efficient and cost-effective method for screening novel vaccine candidates in a natural host model.

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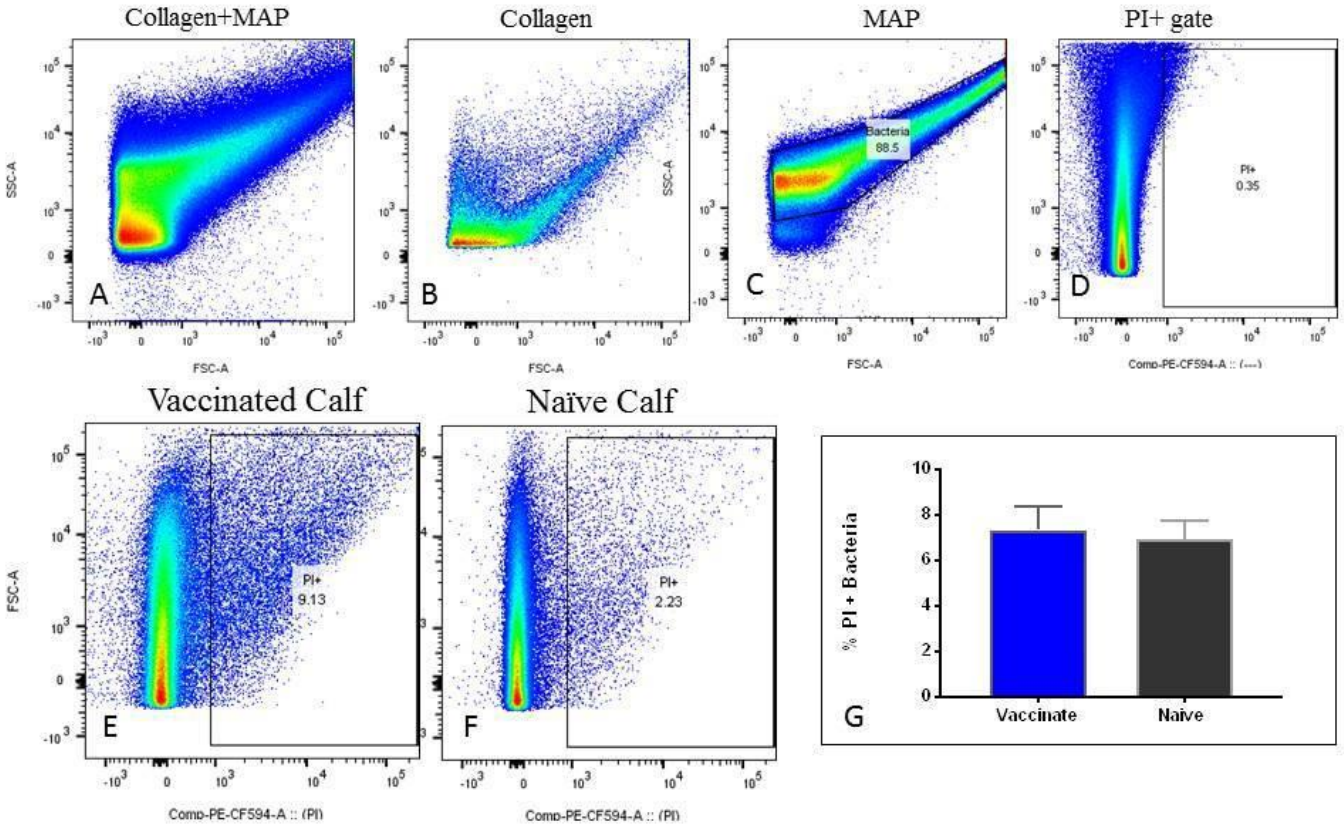


Figure 1: Flow cytometric analysis of bacterial viability 2 weeks post-vaccination. A-D Demonstrates the establishment of gates. **A-** Collagen and MAP. **B -** Collagen only. **C** – MAP only. **D** – Collagen and unstained MAP. **E** – Vaccinated calf. **F -** Naïve calf. **G** – Bacteria from implants (n=9 per group) retrieved from vaccinated calves and naïve calves did not exhibit significant differences. Data are expressed as means +/- SEMs. SSC-A (side scatter area), FSC-A (forward scatter area), PI (propidium iodide), PE (phycoerythrin).

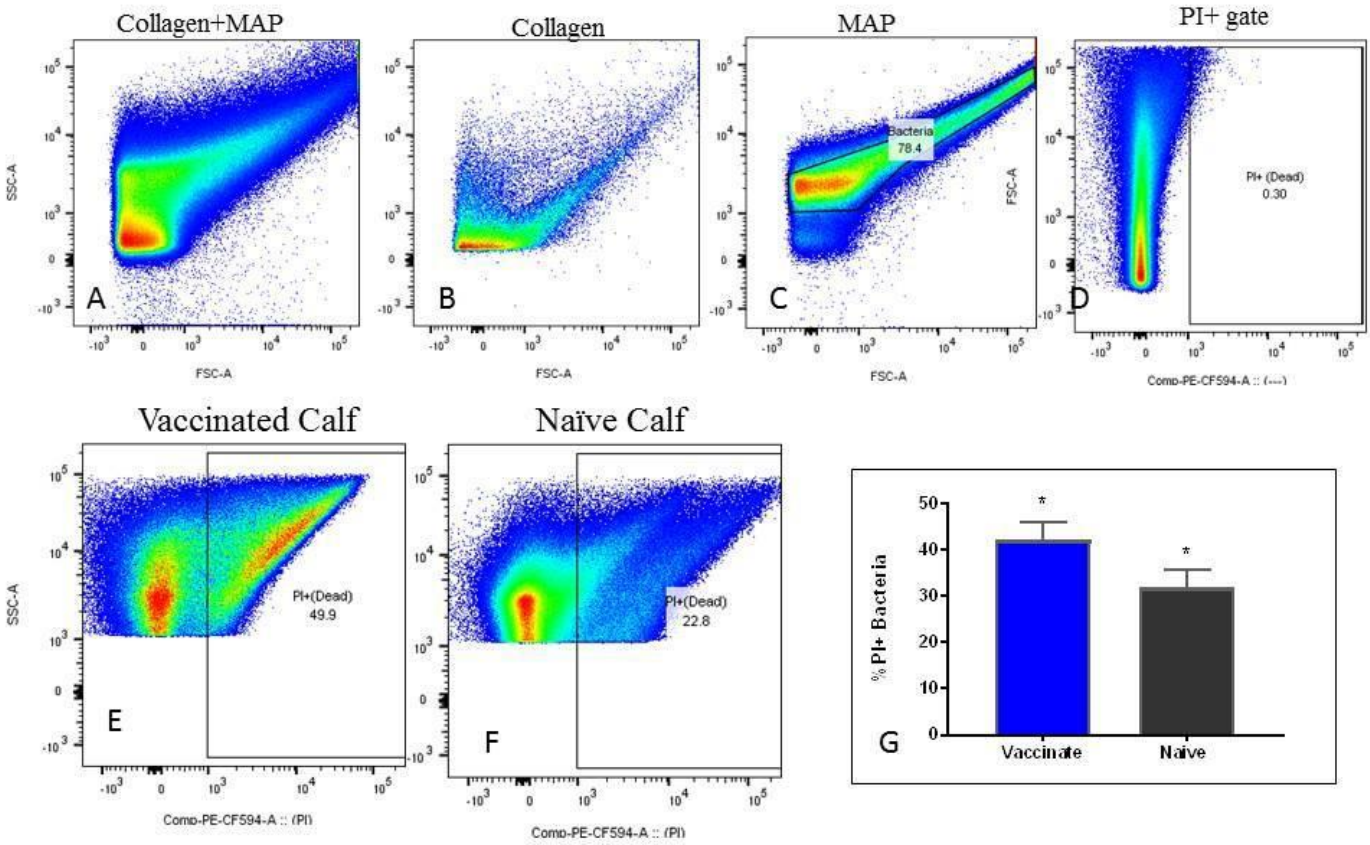


Figure 2: Flow cytometric analysis of bacterial viability 3 weeks post vaccination. A-D Demonstrates the establishment of gates. **A-** Collagen and MAP. **B -** Collagen only. **C** – MAP only. **D** – Collagen and unstained MAP. **E** – Vaccinated calf. **F -** Naïve calf. **G** – Bacteria from implants (n=9 per group) retrieved from vaccinated calves contained a significantly greater percentage of PI+ (dead) bacteria. Data are expressed as means +/- SEMs. *P < 0.05. SSC-A (side scatter area), FSC-A (forward scatter area), PI (propidium iodide), PE (phycoerythrin).

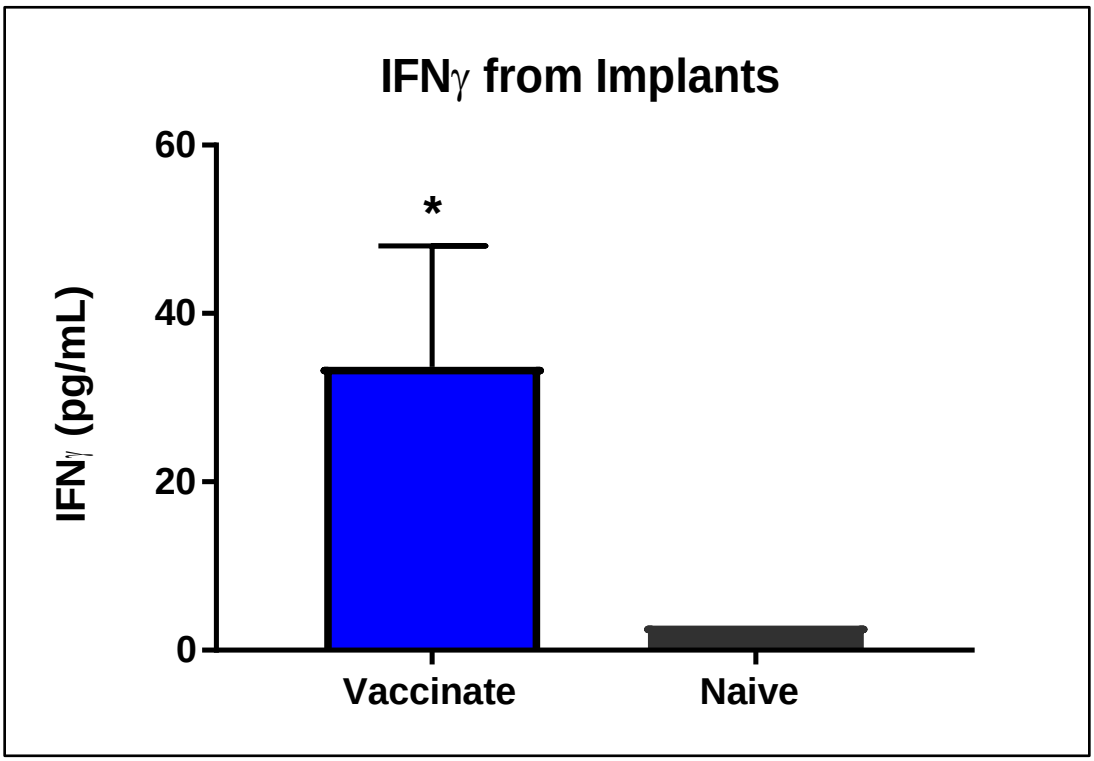


Figure 3: IFNγ levels from retrieved implant collagen at 3 weeks post vaccination. IFNγ levels from retrieved implant collagen 3 weeks post vaccination. Data are expressed as means +/- SEMs. *P < 0.005.

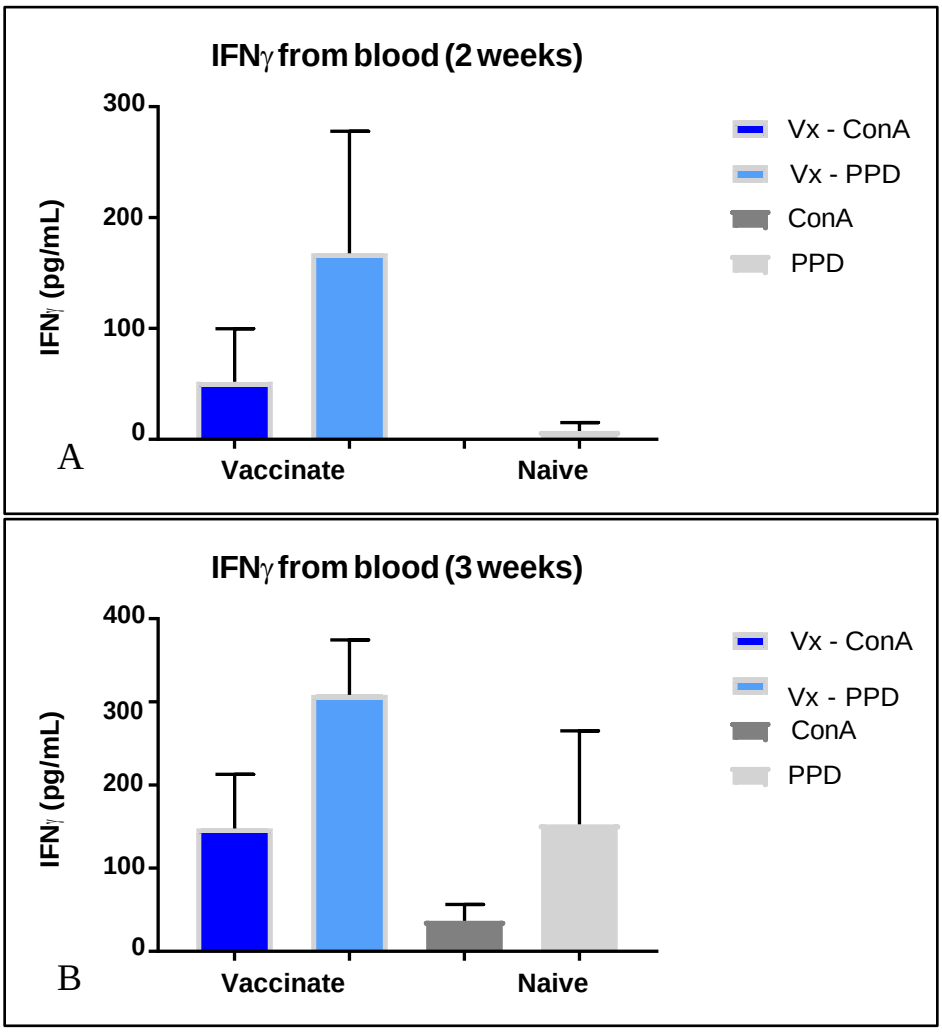
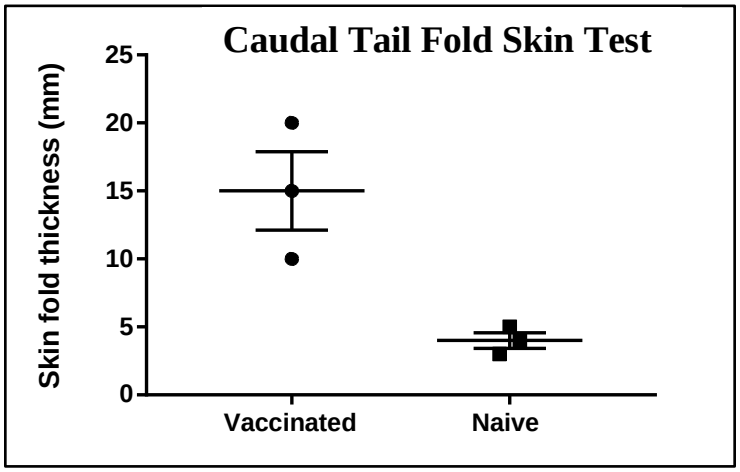


Figure 4: IFNγ levels from stimulated peripheral blood leukocytes. A- 2 weeks, **B** – 3 weeks. IFNγ levels from medium alone (no stimulation) were subtracted from ConA and PPD-J. Statistical differences were not observed between ConA stimulation or PPD-J stimulation of vaccinated and naïve calves. ConA – ConcanavalinA, PPD-J - purified protein derivative-johnin.



Caudal tail fold thickness (millimeters)	
Vaccinated	**Naïve**
15	4
10	5
20	3

Figure 5 & Table 1: Caudal tail-fold skin test thickness. Skin test thickness was measured once at the end of the project. Statistical differences were not observed between vaccinated and naïve cattle.

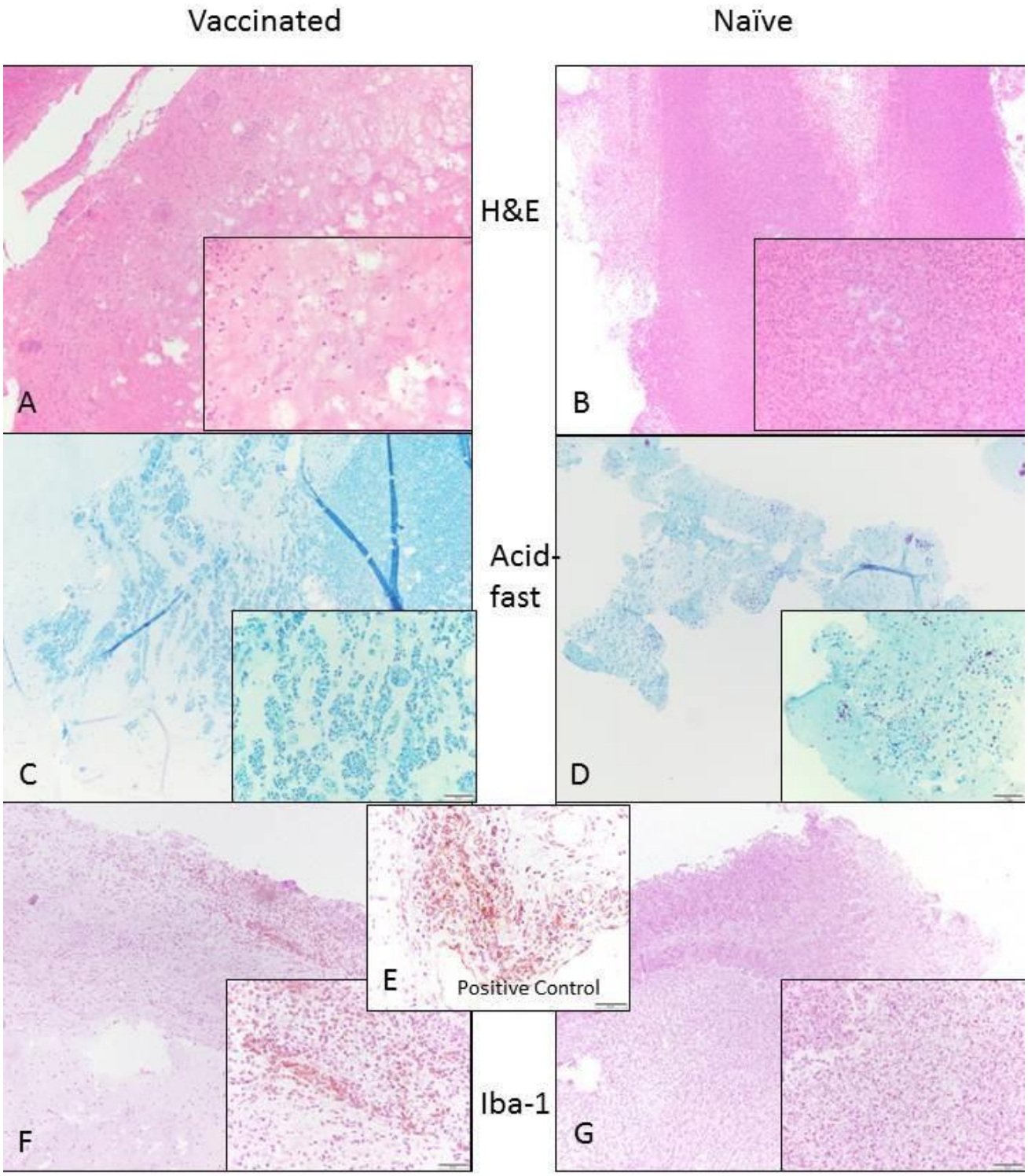


Figure 6: Histopathologic examination of implants. All figures are observed at 100x magnification with a 400x magnification inset. **A,C,F** – Vaccinated calf**. B,D,G** – Naïve calf. **A,B** – H&E sections. **C, D** – Ziehl-Neelsen acid fast stained. **E,F,G**- Iba-1 (macrophage-specific calcium-binding protein) stained.

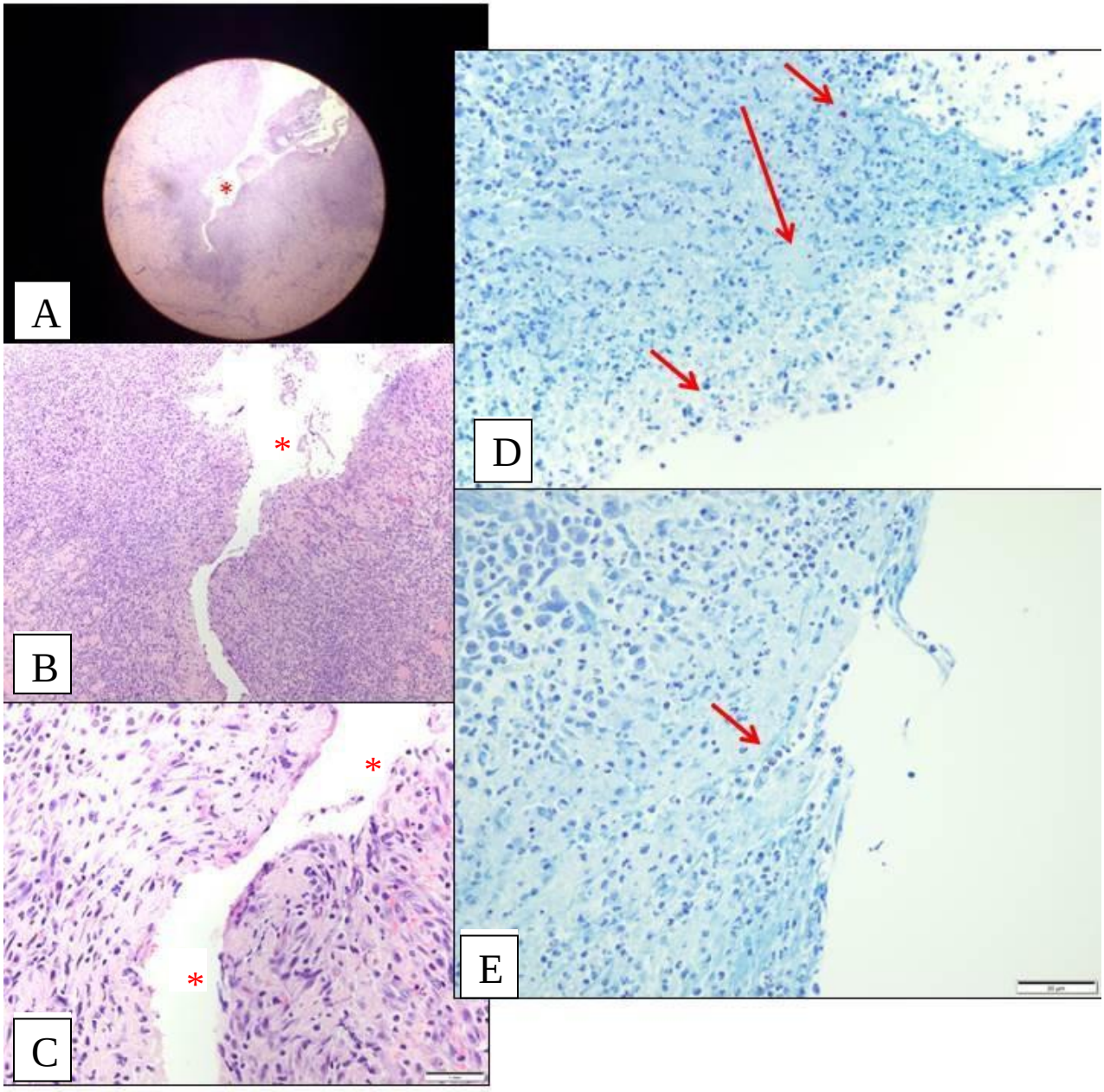


Figure 7: Histopathologic examination of tissue surrounding implant. A-C, H&E. **D-E,** Ziehl-Neelsen acid-fast. **A, B, C**- 20x, 100x, & 400x, tissue surrounding implant. The area where implant was removed is marked with red asterisks. **D, E** – Acid-fast stained tissue surrounding implant at 40x shows subcutaneous bacteria outside of implant. Low numbers of MAP were observed in the subcutaneous of some calves and appeared independent of vaccination status.

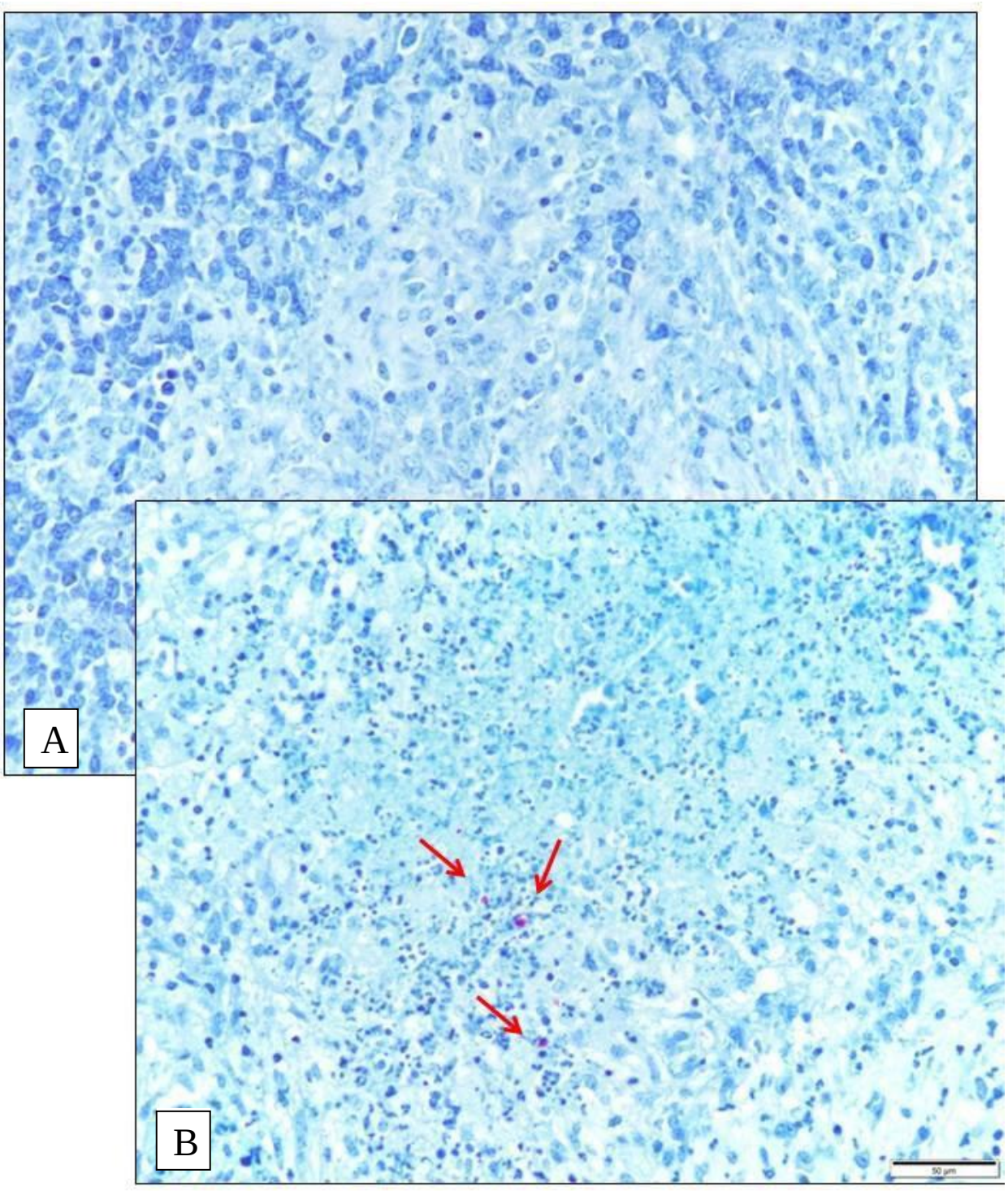


Figure 8: A, B – Histopathologic examination of prescapular lymph node. 400x, Ziehl-Neelsen acid-fast stained, prescapular lymph nodes. **A-** Lymph node without acid-fast bacteria. **B-** Red arrows show intracellular acid-fast bacteria within the prescapular lymph node. Low numbers of MAP were observed in the prescapular lymph nodes of some calves and appeared independent of vaccination status.

CHAPTER 5: GENERAL CONCLUSIONS

Summary

The central hypothesis of this dissertation was that a self-contained, collagen-containing implant device could be developed to detect vaccination-induced, cell-mediated immunity in the subcutaneous tissue of calves. The long-term, future aim of these combined studies is to use the implant device to efficiently screen novel MAP vaccine candidates. This *in vivo* screening test would rapidly and inexpensively provide knowledge of a vaccine's efficacy in the host species; vaccine candidates exhibiting evidence of efficacy could then be selected for more extensive vaccine challenge studies. A better understanding of *in vivo* cell-mediated immunity in response to MAP immunization is essential for the development of a novel vaccine screening test. In all of the studies presented, Mycopar® vaccinated bull calves were compared to unvaccinated, control calves.

In Chapter 2, our specific aim was based on a proof-of-concept study that focused on the development of a platform for *in vivo* detection of cell-mediated immunity in cattle. Our strategy was to design an easily removable device that can house MAP antigen and collect immune parameters while placed in the subcutaneous tissues. The principle for this design approach is that such a device will measure immune parameters at the site of antigen exposure in a living calf and represent a more accurate reflection of the true immune response observed in infection and vaccination.

This first study was a small (n=4) pilot study and focused on the implant design, implantation procedure, collagen processing, and immune parameter retrieval. The design of the device and procedure for implantation is described thoroughly in Chapter 2. Histological sections

were examined along with the concentrations of cytokine from the retrieved collagen. The results of the study provided evidence that MAP-specific *in vivo* measurements can be achieved with the use of this implant device. Histological analysis revealed a lack of cellular infiltrate in naïve calves as well as the vaccinated calf not challenged with MAP antigen (PPD-J). This demonstrated antigen dependent immune cell recruitment into the implant. It showed the collagen's capacity for cellular migration. We hypothesized an elevation in IFNγ and decrease level of IL-10 in the retrieved collagen. As expected, IFNγ was only detected in the antigen-containing implants that were placed in vaccinated calves. IL-10 did not exhibit antigen or vaccine-dependent trends. Overall, the advantage of this novel device is the ability to measure multiple immune parameters upon collagen retrieval to more accurately assess the *in vivo* immune response.

In Chapter 3, we built on our initial proof-of-concept investigation by evaluating the implant device's ability to consistently and repeatedly measure immune parameters in a longitudinal study comparing naïve and vaccinated calves. Our hypothesis for these studies was that the implant device will detect vaccination-induced cell-mediated immunity in vaccinated calves that differs from the immunity observed in naïve calves. To test our hypothesis, an 8 week longitudinal investigation was conducted to evaluate the device's success at identifying vaccinated calves from naïve calves. The implants contained either collagen with saline only or collagen with MAP antigen, and cytokine (IFNγ and IL-10) levels within the implant were measured at 0, 2, 4, 6, and 8 weeks post-vaccination. IFNγ levels in the MAP antigen-containing implants placed in vaccinated calves were significantly higher than the implants retrieved from naïve cattle at weeks 4 and 6 post-vaccination. These responses paralleled the responses observed in MAP-stimulated PBL from vaccinated calves. Significant differences in IL-10 levels

between the implants within vaccinated and naïve calves were not observed. The intradermal caudal fold test (CFT) demonstrated false-positive rates when one of our calves reacted to PPD-J prior to vaccination. Therefore, our implant device was capable of identifying each of the 3 vaccinated calves, suggesting an increased specificity over the intradermal CFT using PPD-J. Overall, the implant device consistently detected *in vivo* immune parameters and evidence showed this device may have a greater specificity than standard intradermal skin tests.

Our final aim, investigated in Chapter 4, involved an adaptation of the previously designed implant device; in Chapters 1 and 2, the MAP antigen, PPD-J, was used in the collagen matrix. In contrast, our final project used live MAP bacteria and explored the device's potential as a bacterial killing assay. The research in this chapter used the implant device as a platform for assessing bacterial viability in Mycopar® vaccinated calves.

In this study, collagen and MAP-containing implant devices were placed in the subcutaneous tissues of calves divided into two groups, naïve and vaccinated calves, at 14 and 20 days post-vaccination. After 5 days, implants were retrieved from the subcutaneous tissues and collagen was processed for evaluation of IFNγ cytokine levels and flow cytometric analysis of bacterial viability. Three weeks post-vaccination, flow cytometric analysis of the bacteria removed from collagen demonstrated a significantly greater percentage of propidium iodide (PI) stained-MAP in the vaccinated calves compared to naïve calves. This indicated an enhancement of bacterial killing within the group of vaccinated calves. Collagen from vaccinated calves at the three week time point also demonstrated a significantly higher production of IFNγ than collagen from naïve calves.

Using flow cytometry to assess bacterial viability, this investigation demonstrated the device's potential as a bacterial challenge model. A subcutaneous bacterial challenge model

could prove to be an efficient and cost effective method for screening novel vaccine candidates in a natural host model.

Future Directions

Various methods are currently recommended in the control of Johne's disease in our cattle herds such as improvement of biosecurity methods and better test-and-cull management strategies [1, 2]. Although such strategies have been implemented, MAP infection continues to exist and cause substantial economic losses across the United States and worldwide [3-5]. Vaccination programs may contain the greatest potential for elimination of MAP infection in ruminants. One of the largest obstacles in the development of new vaccines is the poor correlation between initial screening tests and secondary evaluation methods in the sheep and cattle natural host models [6-8]. A better understanding of how the host immune system responds to local antigen exposure may substantially accelerate our progress in developing a vaccine candidate capable of mounting a fully protective immune response.

In Chapter 2, we developed a removable subcutaneous device that housed MAP antigen while allowing migration of cell-mediated immune parameters into the collagen for collection and analysis. In this study, the MAP antigen chosen for placement in the collagen was PPD-J. In our research, a false-positive intradermal CFT was identified when one of our calves responded to the PPD-J prior to MAP vaccination. This is similar to results seen in other vaccination studies in which intradermal skin testing has been shown to have variable false-positive rates due to sensitization and cross-reactivity of environmental mycobacteria [9-12]. Future studies may benefit from inclusion of antigens specific to individual species of mycobacteria. Evaluating MAP candidate antigens for improved specificity and superior immune protection is an active

area of research in the field of mycobacteria [13-15]. It is possible with the use of one or more MAP-specific antigens that this implant device may be able to differentiate vaccinated calves from subclinical and clinically infected cattle.

In Chapter 3, we conducted a longitudinal evaluation of *in vivo* immune responses collected from the collagen within our subcutaneous implant device. The cytokines included in this dissertation included IFNγ and IL-10. Levels of IFNγ within implants from calves vaccinated with Mycopar® were higher than those from naïve cattle beginning at week 2 post-vaccination. There were no significant differences between vaccinated and naïve calves in IL-10 levels within the implants at any time point in the study.

Numerous studies investigating cytokine profiling in MAP infection has focused on peripheral blood immune responses, as well as sites of infection such as ileum and lymph node [16, 17]. There have been several papers focused on cytokine expression profiles in cattle, sheep, and goats [18-20]. One example is the extensive work by Coussens et al. and their examination of the gene expression of 13 different cytokines in PBMCs, intestinal lesions, and mesenteric lymph nodes of MAP-infected and naïve cattle. These findings provided evidence for the enhancement of anti-inflammatory cytokines and suppression of pro-inflammatory cytokines when cells were stimulated with MAP antigen. Minimal literature exists on the immune responses, cellular infiltrate, and cytokine profiles observed within the intradermal CFT site in cattle. However, some human studies have used immunohistochemical stains to evaluate cytokines at several time points post-PPD intradermal injection in BCG-vaccinated individuals [21]. Although the IFNγ levels within the implants throughout this dissertation proved to be consistent and significantly different between experimental groups, assessing a larger number cytokines could provide a better understanding of the *in vivo* cytokine levels in MAP-induced

DTH reactions. A more extensive profile evaluating the expression of multiple cytokines at the DTH site may prove beneficial to future investigations utilizing this implant device.

In Chapter 4, we adapted the implant device for use as a bacterial challenge model. Our results showed a significantly greater percentage of propidium iodide (PI) stained-MAP in the vaccinated calves compared to naïve calves, indicating enhanced bacterial killing within the vaccinated calves. The enhanced bacterial killing observed in the collagen from vaccinated calves also contained significantly greater concentrations of IFNγ. It is well known that IFNγ is produced by natural killer (NK) cells, NK- T cells, CD4 Th1, and CD8 cytotoxic T lymphocyte (CTL) effector T cells [22]; however, only low numbers of lymphocytes were observed in the histologic sections. Further research is necessary to identify the cells producing the elevated IFNγ observed in vaccinated animals. It is plausible that the lymphocytes, albeit in low numbers, are producing high levels of IFNγ in vaccinated calves. Alternatively, some human studies have shown neutrophils are a source of IFNγ during acute infections, and they should be ruled out as a source for the IFNγ production [23, 24]. Irrespective of their role in IFNγ production, neutrophils have been shown to play a protective role in M. *tuberculosis* infection in mice [25], and future studies are needed to further evaluate the role neutrophils may play in the immune response that has been repeatedly observed in the histologic sections of implant collagen.

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