Vaccine Immunity to Attenuated Coccidioides

Specific Aims

Coccidioides posadasii and *Coccidioides immitis* are the causative agents of coccidioidomycosis (valley fever or VF). *Coccidioides* spp. are endemic to the U.S. southwest, especially the deserts of Arizona and southern California including the San Joaquin Valley (2). Inhalation of a spore (arthroconidium) usually results in a respiratory syndrome that is self-limited and followed by life-long resistance to illness from re-exposure (3). However, illness from the self-limited infection accounts for nearly a third of all community-acquired pneumonias in Arizona and commonly lasts for many weeks to many months (4-9). Also, in some patients, infection spreads to other parts of the body such as skin, bones, and brain, resulting in 161 deaths annually (10). Annual inpatient costs are over $300 million (11, 12). Outpatient medical cost and lost productivity only increase this figure (13). Within the endemic region, the per capita impact of Valley Fever is comparable to that of polio prior to effective vaccination (14). The only effective treatments for these persons are with protracted administration of anti-fungal drugs. These are expensive, can have serious side effects, and are not curative (15). In addition to humans, dogs in the endemic area also contract VF and develop serious disease, the rate and range of which is similar to humans (16).

We have created a strain of *C. posadasii* (Cp) that lacks the CPS1 gene (*Δcps1*) and causes no disease in either immunodeficient (SCID) or wild type mice. Based upon these findings, the University of Arizona has reduced laboratory containment for *Δcps1* from BSL3 to BSL2. Despite this virtually complete attenuation, inoculation of mice with arthroconidia of *Δcps1* results in exceptional protection from lethal pulmonary infection with fully virulent wild-type Cp. The vaccine dose of *Δcps1* used in these studies was <0.1% of the weight of a killed whole cell vaccine dose (17). Although the killed vaccine protected mice, such doses in humans were toxic and ineffective (18). This protection is also greatly superior to our best recombinant chimeric vaccine as shown below in Figure 1. Moreover, in contrast to the several technical and financial challenges posed by formulating a recombinant vaccine (14), manufacturing a spore vaccine should actually be affordable.

The long term programmatic goal is to produce, validate, and market a safe and effective vaccine that protects dogs against naturally occurring coccidioidal infection. We believe this is feasible and much more likely to be licensed than to seek licensure from the FDA for human use since there is no precedent for determining adequate safety of a live eukaryotic vaccine. If the vaccine is found to be safe and effective in canines, we believe the case will be that much stronger to then address its possible development for humans. Before embarking on the manufacturing and formulation tasks that a commercial vaccine will require, we intend to use *Δcps1* that we currently have available to conduct prototype studies that would then guide future product development.

Specific Objectives:

Aim 1. Determine the differences, if any, between immune responses and resulting protection following either intranasal or subcutaneous administration of *Δcps1*.

a. We will use an adaptive study design to establish the lowest vaccine dosage that results in maximal protection by each route and simultaneously measure humoral and cellular responses.

b. We will identify among the humoral and cellular immune responses to *Δcps1* vaccination those that best correlate with protection from subsequent infection to serve as surrogate markers of vaccine immunity.

Aim 2. Determine the underlying immunological basis for *Δcps1* protection by cell purification, adoptive transfers, and cytokine evaluation.

Significance

Of the estimated 150,000 annual VF infections in humans, two-thirds are subclinical. Of the remainder, most have a subacute respiratory illness which, over many weeks, eventually resolves without treatment. For the small number who do not control their initial infection, complications are numerous and serious as detailed above. With or without illness (even those with complicated first infections), virtually all persons develop life-long immunity to a new illness from an exposure to another arthroconidium (3). This observation has for decades attracted physicians and scientists to the goal of preventing VF by developing a preventative vaccine (18-21). Besides humans, coccidioidomycosis occurs in many other species including dogs, cats, horses, sea otters, walruses, South American camellids, nonhuman primates, and zoo animals housed in the endemic area. In these species, it produces similar pathology (16). Because of these similarities, were a preventative vaccine successfully approved for canines or any other animal species, it would have potentially profound implications for following a similar vaccine strategy for humans.

In the fungal plant pathogen, *Cochliobolus heterostrophus*, a pathogen of corn, deletion of the *CPS1* gene, resulted in infections that produced smaller lesions on plants (22). We therefore hypothesized that *CPS1* deletion in Cp might reduce its pathogenicity in animals. This indeed proved to be the case as detailed below. As also shown below, immunization with *Δcps1* resulted in nearly complete protection in mice to lethal respiratory infection with virulent wild-type Cp. The virtually complete attenuation of *Δcps1* coupled with its capacity to stimulate highly protective immunity is the basis for the proposed studies.

Innovation

This proposal is innovative in that it uses genetic information from a plant pathogen to attenuate an animal pathogen and then uses it to provide proof of concept for a preventative vaccine. We believe that this live attenuated vaccine candidate could safely be used clinically to prevent coccidioidomycosis. If so, this would be the first live attenuated vaccine approved for a human eukaryotic pathogen. Because safety of such an approach has no precedent, we anticipate that the FDA would have considerable, possibly insurmountable difficulty approving this vaccine for human application. To avoid this regulatory concern, we are using the innovative strategy of developing the vaccine for a canine application as a proof of safety and efficacy in field use and with naturally acquired infection. Also, the studies in this proposal will employ adaptive study designs. These have been increasingly useful to reduce clinical trial sample sizes (23, 24) and will allow us to determine optimal vaccine dosing in mice and requires fewer animals than would otherwise be needed.

Approach

Overall hypothesis: Some genes important for fungal virulence in some phytopathogens (for example, CPS1 in *Cochliobolus heterostrophus* for corn) are also important for virulence of a medically important fungal pathogen (for example, Cp).

The function of CPS1 has not been established in either *C. heterostrophus* or Cp, but based upon homology it may be an acyl-AMP ligase-like enzyme (22). The Cp CPS1 sequence is closely related by the entire sequence to CPS1 in *Uncinocarpus reesii* and the medically important *Trichophyton rubrum* (Jock itch) and *Arthroderma gypseum,* suggesting a role of CPS1 in Cp in its relationship with the mammalian host. Also based upon sequence analysis, we hypothesized that it might play a regulatory role in pathogenesis. We have performed RNAseq experiments on RNA isolated from *in vitro*-grown spherules of wild-type Cp and *Δcps1*. In *Δcps1*, as expected, the CPS1 transcripts were abolished. Interestingly, we also found about 55 genes either significantly up or down regulated as compared to the transcriptome of wild-type Cp. These preliminary findings are consistent with a possible regulatory function for CPS1 in Cp.

We have found *Δcps1* to be highly attenuated in mice. Intranasal inoculation of 50 wild-type (WT) arthroconidia of Cp is lethal in 100% of BALB/c and C57BL/6 (B6) mice (25). In contrast, inoculation with up to 4,400 *Δcps1* arthroconidia in B6 mice caused no deaths, no weight loss, and no necropsy evidence of lung disease 28 days after infection. Challenge of BALB/c mice with 25 million *Δcps1* spores showed resolution of infection histologically after 7-10 days. As a further test of the attenuation of *Δcps1*, we inoculated NOD-*scid*-

γc^{null} mice intranasally with 1,080 spores. These mice did not become clinically ill during 14 days of observation. We recovered viable fungus from the lungs of only a single NOD-*scid*-γc^{null} mouse by culture, but no spherules were seen by serial histological section through the entire lungs of several additional mice, even with a *Coccidioides*-specific immunohistochemical stain. When we inoculated mice with 10,000 spores, we could identify only low numbers of spherules in the lung on day 3 post infection.

The inoculation of *Δcps1* stimulates a highly protective response in mice. As shown in Figure 1A, intranasal infection of BALB/c mice is a very stringent challenge as evidenced by no protection from a chimeric antigen vaccine candidate that is highly protective in B6 mice (21). However, 19 or 20 mice vaccinated either intranasally (IN) or subcutaneously (SC) with *Δcps1* survived a lethal dose of wild-type Cp. When unvaccinated BALB/c mice are challenged intranasally, total lung fungal burdens are greater than 10^6 cfu (21, 25). In contrast, *Δcps1*-vaccinated mice showed a median of only 3 cfu (Figure 1B).

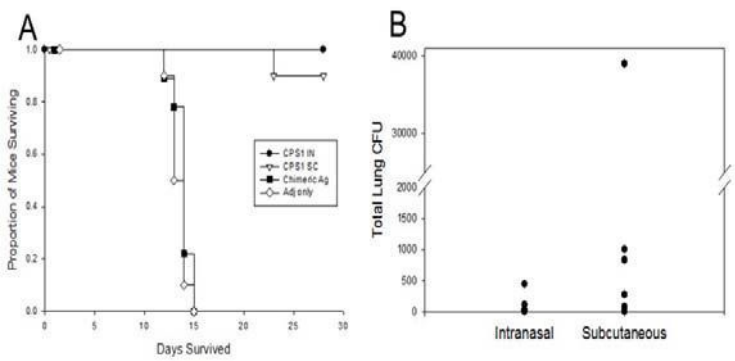

Figure 1. BALB/c mice were inoculated twice with *Δcps1*, a chimeric recombinant protein with adjuvant, or adjuvant only. They were then challenged with a lethal dose (50 spores) of WT *Coccidioides*. Panel A shows a survival curve and panel B shows colony-forming units (cfu) recovered from whole lungs at 28 days post-infection in surviving mice.

It was shown some time ago that T cells from mice vaccinated with formalin-killed spherules can transfer protection from intranasal Cp infection (26, 27). More recently, SC vaccination with another attenuated Cp mutant has resulted in protection and this was associated with T-helper-1 and T-helper-2 cytokines (28). SC vaccination with a live attenuated *C. immitis* mutant produced protection against intraperitoneal WT infection and this was mediated by both CD4 and CD8 lymphocytes (29). Based on these past studies we will concentrate on T cell responses, but not ignore B cell responses. There is no published information about protective immunity that results from IN vaccination with a live attenuated Cp such as *Δcps1*. If there is no difference from what has been observed with SC vaccination, SC is attractive as practical and common in veterinary practice. However, if IN vaccination was in some way superior, understanding the reasons for those differences might be valuable in guiding future vaccine development.

Aim 1. Determine the differences, if any, between immune responses and resulting protection following either intranasal or subcutaneous administration of *Δcps1*.

Hypothesis and rationale- We know from our initial studies that the inoculation of *Δcps1* twice in the airway or subcutaneously can generate protection from lethal Cp challenge. We do not know which immune responses are generated either systemically or locally following inoculation. **Furthermore, we do not know which of those responses mediate protection**. We hypothesize that based on earlier work using wild-type *Coccidioides* (30) that inoculation with *Δcps1* will result in a strong CD4 /Th1/ Th17 (31, 32) and CD8 responses (29). B cell responses are likely to be present and may participate in the resistance to challenge (33). Because our earlier data in *Francisella* suggest that the route of infection influences the response (34), we will explicitly examine the local and systemic responses to *Δcps1*.

In this aim we will test two routes of inoculation – intranasal (IN) to stimulate a mucosal response in the airway and subcutaneous (SC), potentially a more practical route of administration for future clinical vaccination protocols. For each, we will then determine the relationship among the number of required doses of a vaccine and the amount of immunogen per dose.

Approach- All studies will utilize B6 (or BALBc) mice. This will ensure that we can ultimately use all of the available genetic tools that are available on the B6 genetic background (a few like the stat4 knockout are only available on BALB/c and we will use those as required). We will start with a vaccination protocol known to protect: prime and boost two weeks later. The infection challenge of 50 WT IN is delayed for four weeks following the boost to avoid non-specific vaccination benefit that has otherwise occurred with some coccidioidal vaccines, for example those with CpG adjuvants. All mice will be observed for clinical signs of illness (weight

loss, hunched posture, ruffled fur, labored breathing and dehydration). Two weeks following infection, mice will be sacrificed and lung fungal burden quantitated as colony forming units (cfu).

We will use adaptive design. Basically this methodology is rooted in Bayesian logic using hidden Markov chains. This begins with two random doses (they can literally be random) but we would begin with 10,000 (known to produce protection) and ten-fold less of *Δcps1* spores. Controls will include heat killed *Δcps1* as well as UV irradiated *Δcps1* since dead arthroconidia in these numbers are not protective (19). Eight mice in each group are sufficient. We will use four for the protection study and the other four will not be challenged but will be tested for T and B cell responses. This will allow us to determine the type and magnitude of the immune response elicited by *Δcps1*. We will choose the best dose for protection based on reduction of lung cfu and then pick a third and fourth dose to flank the best response(s). For example, if the responses are equivalent, we will vaccinate with 100,000 and 100 spores, 8 mice in each group. If the higher dose is better, we will vaccinate groups with 100,000 and 500 spores. While the responses of any given experiment cannot reach statistical significance the trends themselves will be used to guide the dosing decisions. A final experiment will select a candidate vaccine dose and a sub-efficacious dose with a larger sample size (n=8 mice) to validate the perceived trends from the initial experiments. This approach has been widely used in chemotherapy trials (23, 24), but this is a novel use in a pre-clinical vaccine trial.

The primary outcomes of the final experiment will be the reduction in lung fungal burden. We know from many past coccidioidal studies that enhanced survival always accompanies reduction in lung fungal burden and cfu enumeration is a more robust indicator of vaccine efficacy. This approach will also define the markers of a protective vaccination. We will then establish a dose of *Δcps1* spores that will protect and produce humoral or cellular immunologic responses following a single immunization, if any. Simultaneously, we will be generating the secondary outcome measures of immune responses as follow.

T cell responses will be assayed in two ways. Our initial experiments will use cytokine production by spleen cells, and lung cells. We will use the same methods that we have successfully used to measure cytokine production in *Francisella*-infected mice (34-37). Briefly, cell suspensions are prepared and co-cultured with APC that have been cultured overnight with varying amounts of spherule extracts or recombinant coccidioidal antigens such as we have used previously (21, 38) to allow for antigen processing. These cells (from CD45.1 congenic mice to allow for identification in flow) are cultured with cells isolated from inoculated mice for 18 hours. Supernatants are tested by Luminex array for 20 cytokines. Based on the results of preliminary cytokine assays, we will then test the lymphocytes for intracellular cytokine production (using different cultures, since the cells to be analyzed for cytokine secretion must be treated with Brefeldin A to block secretion) and analyzed by flow cytometry for intracellular cytokines. We have extensive experience in these assays both from mouse spleen and lungs (1, 35, 39-41). An example from *Francisella* infected lungs is shown in Figure 2. It will be important to measure both the total numbers in each leukocyte population as well as the percentage of each that are expressing the relevant cytokine in the lung and spleen. We know that some bacterial pathogens elicit quite different responses depending on the route of administration, and that an attenuated mutant of *Francisella* (ΔclpB), for example, can induce a more Th1 directed response than the WT bacteria (35). It will be important to assess these responses quantitatively.

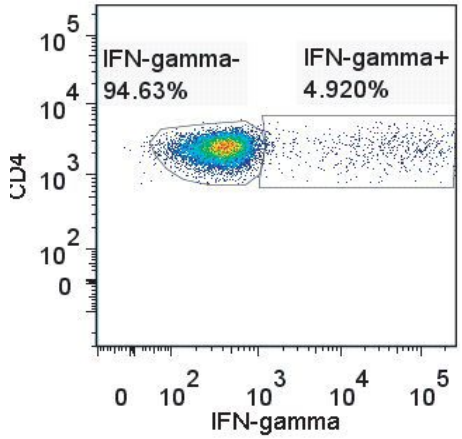

Figure 2. Data from Roberts et al (1). Single $CD4^+$ T cells from the lungs of *Francisella-LVS* mice were isolated on day 7 following infection and stained with anti- CD3,4,and 8 and dump channel antibodies anti- CD11b, B220 and CD45.1. Cells here are gated on $CD4^+$ T cells and anti Interferon-γ following overnight culture with *Francisella* infected CD45.1 APC. Percentages indicate the percentage of $CD4^+$ T cells.

We will also characterize the cellular infiltrate in lung tissue and BALF by Luminex and flow cytometry with methods similar to those for splenocytes. We have extensive experience in this [see (1)]. With a single panel we can assess alveolar macrophages, interstitial macrophages, dendritic cells and neutrophils, as well as lymphocyte subsets.

Antibody responses - We will bleed the inoculated animals before sacrifice and assay the serum and BALF in two ways. First we will develop ELISAs using a complex spherule extract and recombinant coccidioidal antigens for coating wells. We have done this many times (42-45), most recently for *Francisella* [1]. We will then determine the serum dilution that results in half maximal signal. If we detect signal we will perform western blots against Cp spherule extracts and recombinant coccidioidal proteins (25, 46). While there is little evidence that antibody responses are themselves protective, they reflect sensitization and potentially could serve as a surrogate marker of effective vaccination. We will also determine the IgG subtypes of these antibodies in ELISA as they will give clues to the Th subset produced (IgG_{2c} implies Th1 and IgG_1 with Th2). Coupled with the results from the cytokine assays, they add strength to the understanding of the polarization of the *Δcps1* induced responses.

These experiments will characterize the immune response to *Δcps1*, but will not describe the response during the secondary infection with wild-type Cp. Secondary responses following a vaccination can have different requirements from primary responses. For example, IL-17 is required for recovery from primary infection following *Francisella* challenge (39). In contrast to the primary infection, IL-17 is completely dispensable during subsequent challenge (39). Thus, it will be important to examine both the cytokines produced in the *Δcps1* inoculated mice as possible surrogates for vaccine efficacy as well as following wild type Cp challenge which may be those that actually mediate protection. For that reason we will examine the responses following challenge with wild-type Cp and evaluate the same cytokines as above.

Results and Interpretation- While there is currently no data on either the adaptive or innate immune response following inoculation with *Δcps1*, there is substantial evidence that sublethal infection with WT Cp requires a Th1 type response and IFN-γ. While depletion of CD8 cells during the response diminishes protection against an intraperitoneal infection, it is not completely clear from the published work whether only $CD8^+$ T cell or if other $CD8^+$ cells such as $CD8^+$ DC might play a role.

We expect that the inoculation with *Δcps1* will produce a robust Th1 type cytokine response, and a small antibody response. The extent of the antibody response will be important since an antibody immunoprecipitation test by gel diffusion in agar is used as the standard clinical test for diagnosis in dogs and is heavily utilized in humans as well. A potential consequence of immunization might be to distort diagnostic test results. This is a real clinical issue that needs to be investigated in any vaccine development. As shown by our previous work, IN infection with Francisella results in a mixed IL-17/IFN-γ response in the lung, while parenteral immunization results in an IFN-γ only response in the lungs. Both routes result in a predominant IFN-γ response in the spleen.

Based on the response patterns above, we will be able to compare the effect of a parenteral SC inoculation of *Δcps1* to intranasal inoculation, both on the responses in the lungs and in the periphery. Together this will allow us to directly compare the dose and the route of inoculation with two outcomes: 1) the ability to generate local and systemic immune responses and 2) the efficacy of each route and dosing regimen to provide protection against lethal challenge. This information can direct vaccine development toward the most effective route in future clinical trials in dogs.

Problems and alternatives- The major limitation of these experiments is it will be critically important to determine the cytokine/cellular responses that are critical in the resistance to reinfection with WT *Coccidioides*. In order to strengthen that we would need to perform transfer experiments using immune cells from wild-type mice as well as appropriate knockout mice. *In vivo* cytokine ablation experiments would complement those studies. Unfortunately they cannot be accomplished in a two year time frame and an R21 budget.

Based on the lack of protection from previously studied whole-cell killed *Coccidioides* mycelial vaccines, we expect that these preparations will serve as sham controls in the proposed studies and not produce significant protection. To the extent that they create other immunologic responses, they will actually be useful in assessing immune responses that are not correlated with protection. However, if unexpectedly we find significant protection from killed *Δcps1*, we will need to completely reassess the need for a live *Δcps1* and might pave the way for reconsideration of a vaccine for human use as well as for dogs.

Aim 2. Determine the underlying immunological basis for *Δcps1* protection by cell purification and adoptive transfers, and cytokine evaluation.

Rationale and hypothesis- Vaccine development can be completely empiric or can be rationally designed. We know that the immune response to any intact organism is usually complex. The fact that one or another immunologic response occurs does not make it necessary for that response to mediate protection. In this aim we will use the information obtained in the first, focusing on correlates of the secondary protective immune response to drive to the next level- determine those responses that are required for protection. This narrowing of all the responses to those that are required to provide protection will provide critical information about which surrogate markers will be most helpful in phase I clinical trials in canines. While our ultimate goal is to develop a human vaccine, as an intermediate step, the goal of this program is to produce a functional canine vaccine. Achieving this goal will in itself be a major veterinary advance. In addition, it will provide additional evidence of the efficacy and safety of the approach in naturally acquired infection.

Hypothesis- Only some of the immune responses produced following inoculation with *Δcps1* are REQUIRED to protect mice from WT Cp infection. Here, combinations of classical cellular immunology combined with studies in conditional knockout mice will be used to pinpoint the relevant cells and cytokines required following vaccination.

Approach- In the preceding aim we addressed the question of what cells were stimulated to respond by *Δcps1*. In this aim we will address the question of which cells and cytokine responses are necessary for protection.

Our overall approach is very straightforward. We will separate cells from *Δcps1*-inoculated animals. We will address the issue of where the protective cells are localized by using cells from the lung and spleen. Briefly, the isolated immune cells will be fractionated, different fractions will be transferred into naïve recipients, and after 24 hours they will be challenged intranasally with 50 spores of Cp. Fungal loads will be measured at days 6, 10 and 14.

Cell fractionation- We will examine CD4 and CD8 T cells as well as B cells. Cells will be purified initially by untouched cell purification kits from Milltenyi. Graded numbers will be transferred up to 10^7 cells per mouse.

In order to move from the cells to the relevant molecules we will purify cells from reporter strains. Cells from these mice can be identified when a particular cytokine is expressed, usually by coexpression of a fluorescent protein. Currently easily available (from Jackson) are reporters for Interferon-γ, IL-4, and IL-17. By vaccinating these strains, we can enrich for Th1 and Th2 and Th17 cells, respectively, by sorting and isolating cells that fluoresce and then transfer them each into naive non-transgenic recipients. The donor cells will be enriched before transfer and the recipients will be normal. In some experiments knockout mice will be used as recipients to eliminate the contributions for the recipient.

Following transfer, we will re-isolate the transferred cells based on reporter expression and test those cells for their ability to secrete cytokines following *in vitro* re-stimulation with PMA+ionomycin and *Δcps1* infected syngeneic APC. Since each of these T cell subsets secretes an array of cytokines and chemokines, the identification of a Th1 response by marking interferon-γ does not guarantee that interferon- γ is the major cytokine that acts as an effector.

We will identify the cytokines produced following re-isolation for the cells following transfer.

It is possible that the only cells that really are effective are those that produce multiple cytokines (poly functional cells). There is already a hint that polyfunctional cells might be important since interferon-γ knockout mice are not protected by immunization with recombinant protein (47) but are protected by immunization with a live attenuated vaccine (29) and TNFα knockout mice are only partially (29) protected by vaccination with live WT fungus. It is known that many but not all Th1 cells all are able to produce multiple cytokines, and CD8 cells can produce IFN-γ, TNFα, and granzyme B.

Besides the cytokine secretion pattern, it will be interesting to determine the cell surface phenotype of the cells able to mediate protection because the correlation among cytokine secretion and cell surface phenotype is imperfect. Effector memory and Central memory cells differ in their expression of CD62L and can be readily separated based on expression of CD62L. There is substantial interest in the role of central memory and effector memory cells. In influenza infection central memory cells are located in the periphery, but not in the lungs, while effector memory are in the lungs (48). Surprisingly effector memory cells seem to have little effect

on the ability of mice to resist influenza reinfection, while central memory cells are able to mediate protection.

Cytokine(s) expressed by cells that significantly lower fungal loads will be candidates for required mediators of protection. We will use this information to frame the next set of experiments that will directly test the role of each of these cytokines. This will be accomplished by combining cytokine/chemokine receptors knockout and adoptive transfers. Cells from cytokine knockout mice will be inoculated with live or dead *Δcps1* and cells transferred to WT or T cell subset (i.e. CD4/CD8 knockout) recipients. The following day recipient WT mice and also non-vaccinated knockout mice will be infected with Cp and fungal burden determined 6, 10, and 14 days after infection. This study design is preferred to simply infecting vaccinated knockout mice since the immune system of knockout mice can have unanticipated dysregulation. Transfer into a normal milieu will obviate some of these difficulties.

As a final experiment, for cytokine(s) that appear essential for protection, we will inoculate normal mice with *Δcps1* and then treat them with the appropriate anti-cytokine antibody to neutralize these effects. While these are expensive experiments, and are difficult to control for complete neutralization of cytokines, they can provide the final confirmation of the impact of single cytokines.

Outcomes and Interpretation- It would be naïve on our part to expect a single cell and a single cytokine to be solely responsible for protection. In *Francisella* we and others have found a variety of immune responses, both innate and adaptive that contribute variably to both recovery from primary infection, and resistance to secondary challenge. We expect the same here. However, the pattern of response and the contribution of each component will be important for both understanding the immune responses and for the utilization of *Δcps1* as a vaccine candidate in both animals and humans. This data in the R21 will not only provide the correlate of immunity, but also the mechanism of immunity.

Problems, Pitfalls and Alternatives- One technical problem will be the number of cells recovered from different organs. While there will be many fewer lymphoid cells recovered from the lungs of animal following inoculation, we expect, based on our previous experience with other lung infections, that we can recover several million cells from the lungs of each donor. While this will make the transfer experiments challenging, we expect that we will be able to circumvent this. In the worst case, we can use several mice for each recipient. This will remain interpretable since in the tissues where there are large numbers of cells, we will transfer graded numbers of cells to each recipient and can compare efficiency based on cell number and functional ability to secret cytokines.

Our intuition is that several types of cells will contribute to the protection and that we might see partial protection for several. Alternatively and even more complex, we may require more than one cell type. We will control for this by transferring unfractionated cells and paying close attention to the composition of those cells before transfer.

Finally it is possible that any differences we see in efficacy of each organ might be due to the presence of T reg. If this is the case, we will use FoxP3 reporter mice and delete those cells before transfer to determine their impact. While FoxP3 reporters are imperfect, they can be useful. Alternatively we can use FoxP3-DTR mice and delete them with diphtheria toxin *in vitro* before transfer.

Future directions- The goal of this R21 will be to provide the initial data on the immune response to a candidate live vaccine, *Δcps1* that we can use in two important ways. The first will be a better understanding of the roles of different immune responses to *Coccidioides*, an area of great clinical importance in the American desert southwest. Second, it will also provide critical information about a vaccine candidate that will be required as we move this potential vaccine from proof of concept towards a trial in large animals and ultimately to humans.

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