

TEAM 1

Recombinant protein vaccine to prevent coccidioidomycosis in risk groups

Section 1 - Literature Review

Epidemiology

Coccidioides immitis is a dimorphic fungus that lives in the soil of semiarid regions of the southwestern United States. When inhaled in its infectious form, the fungus can cause coccidioidomycosis, also known as Valley Fever. In her review of the process of *Coccidioides* classification, Jan Hirschman discusses the difficulty in identifying the source of Valley Fever infection. The disseminated condition of Valley Fever was first described in 1892 by intern Alejandro Posadas, who was investigating cutaneous lesions on a soldier. After biopsy, the intern misdiagnosed the specimens as a protozoan, because they resembled *Coccidia*. *C. immitis* remained an elusive pathogen to identify, as mold cultures taken from patients were discarded as contaminants until 1900 when William Ophuls and Herbert Moffitt successfully retrieved the organism from nodules on inoculated rabbits. Following the identification of *C. immitis*, further investigation rapidly elucidated the nature and life cycle of the fungus and led to virtually complete classification by 1945 (Hirschman 2007).

Cases have been reported in drastically increasing numbers. One theory for the increase in the incidence of this soil born disease is the urbanization of semi-arid regions in the southwestern United States since the late 1800s. The combination of this agricultural, residential and commercial development disturbed previously uncultivated soil and increased the diffusion of the infectious form of *C. immitis*. Valley Fever is endemic to the southwest United States and regions of Central and South America. Until recently, semiarid regions between 40-degree northern and southern latitudes in the western hemisphere were the only locations where cases of coccidioidomycosis had been identified. The regions in discussion are normally characterized as having climates noted for low rainfall, hot summers, few winter freezes, and alkaline soil. The lower deserts in Arizona and the Central Valley of California have the greatest prevalence of the disease, but Nevada, New Mexico, and Texas are affected as well (Fisher 1988).

In recent years, reports of coccidioidomycosis have risen from all regions of the world ranging from: Australia, New Zealand, the United Kingdom, Israel, and India, with the number of countries reporting the disease increasing yearly. It is thought that the spread of the disease is caused primarily by travel and the cultivation of the soils. In the year 2000 there was nearly 28 million travelers to Arizona and of these, nearly 1 million were international travelers (Cox and Magee 2004). While coccidioidomycosis is not transmitted person to person, resilient arthroconidia can survive in soil or dust trapped on clothing, luggage, shoes, or other materials traveling with visitors leaving endemic areas, which could allow for the spread of the pathogen, and account for the unusual locations of recently reported cases.

Life Cycle

As previously mentioned, climate plays a major role in the number of outbreaks of *C. immitis*. In addition to general climate conditions previously described, a seasonal correlation between increasing reported cases and winter months exists in Arizona. Dust storms, earthquakes, drought and other environmental events lead to dust circulation and dispersal in the air. Until recently, it was believed that *Coccidioides immitis* was the only form of the *Coccidioides* fungus. There has been recent discussion about a potentially new differentiation of the fungus by region, with *Coccidioides immitis* existing in the California areas, and *Coccidioides posadasii* existing in Arizona. There has been no official designation, so for the purposes of this discussion, the pathogen will be referred to as *C. immitis*. As previously mentioned, the fungus is dimorphic, with a saprophytic and parasitic phase. Within the soil, *C. immitis* exists in the saprophytic phase. The fungus is released as endospores from mammalian hosts such as humans, dogs and horses. This usually occurs as a release from an animal carcass. The endospores form tubular structures as they grow into the filamentous mycelia. The mycelia grow in the soil, forming arthroconidia, which are cells that grow along the mycelia. Wind can pick up the arthroconidia with dry topsoil and allow the pathogen to spread to different areas, or to be inhaled by a mammalian host. Once *C. immitis* enters the lungs of a mammalian host, the arthroconidia enlarge and become spherules, which endosporulate and release hundreds of spores into the lungs. Upon release, the spores can cause secondary infection within the host by re-entering the saprophytic phase of the fungal life cycle. Spores can also bypass the saprophytic phase by transforming immediately into spherules. Secondary infection can lead to the formation large granulomas in the lungs or even dissemination of the pathogen throughout the body. The nature of the *C. immitis* lifecycle allows it to be infectious but not contagious (DiCaudo 2006).

Pathogenicity and the Immune Response

Arthroconidia transition to the parasitic cycle in the alveolar clusters of the lungs. Alveolar macrophages and dendritic cells take up the pathogen through Toll-like receptors that induce the NF κ B signaling pathway for the release of pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8, IL-12 and IL-18. This primary pulmonary infection, resulting in about 40% of reported cases, can produce influenza-like symptoms characterized by cough, fever, chest pain, headache, fatigue, chills, malaise and anorexia (Cox and Magee 2004). Asymptomatic cases usually remain subclinical, but can be detected by a coccidioidin or spherulin skin test. Patients experiencing symptoms can receive chest radiographs and sampling of bronchoalveolar lavage fluid. The chemotactic factor IL-8 recruits polymorphonuclear leukocytes (PMNLs), most notably neutrophils, to the site of infection. Macrophage-derived IL-12 and IL-18 also recruit Natural Killer (NK) cells, although their anti-coccidioidal activity is less defined, perhaps carried out by cytotoxicity. Macrophages and neutrophils phagocytose infecting spores for destruction by respiratory burst and phagosome-lysosome fusion (Cox and Magee, 2004). The virulence mechanisms of *C. immitis* are designed to overcome and modulate the host immune response for pathogen survival. At a diameter of 40-60nm, spherules are simply too large to be phagocytosed and uptake and destruction of released endospores is inefficient. *C. immitis* induces activation of Arginase I in macrophages, which competes for the inducible nitric oxide synthase (iNOS) substrate pool of L-arginine. Arginase I converts L-arginine to L-ornithine, a substrate important for fungal growth and

metabolism. Growing spherules also produce and shed immunogenic spherule outer wall (SOW) components that are engulfed by antigen presenting cells which consequently induce a Th2-biased immune response where B cells proliferate and produce coccidioides specific antibodies. Expression of SOW components sharply decreases upon release of endospores, limiting the opportunities for opsonization and phagocytosis during the mature spherule stage where this is problematic (Hung, Xue, and Cole 2007).

Furthermore, Th2 cytokines such as IL-4 and IL-10 induce expression of Arginase I in macrophages, reducing the efficiency of destruction by respiratory burst (Cox and Magee, 2004). Urea, a byproduct of Arginase I activity is further metabolized into ammonia and carbonic acid which eventually creates an alkaline environment that prevents phagosome-lysosome fusion and exacerbates virulence and tissue damage if released into the extracellular milieu. Several experiments have demonstrated the absolute need for a cell mediated immune response. This includes the release of cytokines such as IL-2 for T-cell proliferation, and TNF- α and IFN- γ for macrophage activation promoting phagosome-lysosome fusion and respiratory burst (Hung, Xue, and Cole 2007). Accordingly, low levels of Th1 cytokines and high titers of IgA and IgG are correlated with disseminated coccidioidomycosis. Nevertheless, a balanced Th1 and Th2 response involving cellular and humoral immunity is important to control and remove the infection (Hung, Xue, and Cole 2007). An ever growing subset of cases have demonstrated extra-pulmonary dissemination and infection reaching the skin, subcutaneous tissues, bone, meninges, lymph nodes, spleen, liver, kidneys, pleura, the central nervous system and other tissues, excluding the gastrointestinal tract. Multifocal dissemination is usually associated with a 50% mortality rate (Cox and Magee, 2004).

Populations with higher risk of contracting coccidioidomycosis include construction workers, landscapers, farmers, ranchers, and others in occupations that cause them to spend long periods of time exposed to disturbed soil and blowing dust (CDC, 2005). In addition, immunocompromised individuals, especially those with deficiencies or mutations associated with T-cell receptors, CD4 Th1 cells, or the cytokines associated with a cell mediated immune response (as described above), are more susceptible to disseminated disease. Infections in various ethnic groups have also demonstrated an increase in the risk for developing disseminated disease. These ethnic groups include African Americans and those of Filipino descent. This may be a result of a genetic tendency to proliferate an overwhelming B-cell response, polarizing the immune system and inhibiting the necessary Th1 and Tc cell response (DiCaudo 2006)

Previous Vaccines

Research toward creating a vaccine against *C. immitis* has been in progress for over 100 years. A wide range of vaccine designs have undergone trials, including: viable cells, nonviable cells, cellular extracts, isolated protein antigens, recombinant protein antigens, and most recently fungal DNA. The first vaccines against *C. immitis* were live cells. While these appeared to provide some level of protection, the pathogen was able to spread to other parts of the body. Vaccination with *C. immitis* attenuated with different methods was also attempted, but the fungus demonstrated an ability to renew its virulence

in vivo. In recent years, efforts to develop a viable cell vaccine have been abandoned due to the high risk factors associated with this method. (Cox and Magee 2004)

Research has since shifted to killed whole cell vaccines. In comparing the effectiveness between vaccination with the infectious arthroconidia form and the parasitic spherule form of the fungus, it was found that spherules were most protective. This provides evidence that there is some antigen commonality between the saprophytic arthroconidia and the parasitic spherule phases of the fungal lifecycle. The difference in effectiveness, therefore, may not be due to a difference in antigens present between the forms, but instead a difference in quantity of antigens present. A vaccine containing formalin-killed spherules (FKS) achieved the level of clinical human trials, where it ultimately failed to demonstrate true immune protection. The failure was attributed to the toxicity of the FKS vaccine, which made it impossible to administer in doses large enough to be immunogenic without inducing toxicity. (Pappagianis, D. 1993)

In order to overcome the issue of toxicity, attempts at isolating less toxic antigen from whole cells have been conducted, including extracts from whole spherules. The most notable of these was the 27K extract (so named because the lysate of whole cells was centrifuged at 27,000 x g), which provided protection among most mice strains. It failed to induce protective immunity in at least one strain of mice, and had not proceeded beyond mouse studies as of 2004. Instead of focusing on the extracts, researchers have been more interested in isolating the specific antigens they contain. (Zimmerman et al 1998)

Many protein and peptide antigens have been defined, both in the 27K and other spherule extracts, and vaccine attempts with just these proteins have shown some positive results. Perhaps one of the most well-defined protein antigens from *C. immitis* is the antigen 2/proline rich antigen (Ag2/PRA). Once thought to be two different proteins, it was discovered that gene PRA lies within the gene coding Ag2, and they are now regarded as one protein antigen. Another promising protein antigen is *Coccidioides* specific antigen (CSA). In discovering the gene sequences for these proteins, researchers have been able to produce them as recombinant proteins, having less negative side effects (such as pain or hypersensitivity) when used in mouse vaccination. Vaccination of mice with a combination rCSA and rAg2/PRA was shown to increase survival of mice challenged with arthroconidia by 90 percent, demonstrating the potential of multivalent recombinant protein vaccines to maximize the induction of protective immunity while minimizing negative side effects. DNA vaccination work is extremely preliminary at this point, both for this pathogen and for use in humans in general, and will not be discussed further here (Cox and Magee 2004).

Current Treatments

Current treatments for coccidioidomycosis involve the administration of various types of anti-fungal medications or surgical debridement. The specific antifungal medication prescribed as well as the duration of treatment also varies depending on the type of infection. Since one half to two-thirds of cases are asymptomatic or subclinical, most people who contract Valley Fever need no treatment because primary infection usually

induces lasting immunity to secondary infections (Galgiani 2000). However, in the remaining cases, there is a significant risk of progressive pulmonary disease, disseminated disease, and mortality. For this reason, a patient with a diagnosed case of coccidioidomycosis will have individual therapy tailored to the level of disease. Duration and choice of treatment depends on the severity and speed of disease progression. Some specific antifungals used to treat coccidioidomycosis are amphotericin B, ketoconazole, and fluconazole. In cases of swiftly progressing infection, amphotericin B is most often chosen in early treatment whereas less acute cases are usually treated with an –azole (Galgiani 2000). Surgical debridement is also an option for patients who present with large nodules or progressive disseminated disease. Success of treatment depends largely on diagnosis prior to acute pulmonary progression.

Some disadvantages of antifungal treatments are their costs and side effects. Amphotericin B in particular is associated with rashes, headaches, abdominal pain, and tachycardia among others (RX List, 2007). The high cost of antifungal medication is also significant. One year of treatment can cost from \$5000-\$20,000. Patients with disseminated disease can incur even higher costs. Surgery also has its individual risks and high cost depending on the procedure, as it is an extremely invasive solution compared to other treatments (Galgiani 2000).

The effectiveness of early diagnosis is often compromised by the lack of a consistent, reliable diagnostic test for early infection. This is because current diagnostic testing still relies heavily upon attaining fungal cultures from the patient, which are often undetectable until the infection has become severe. Current efforts in developing a more effective diagnostic test involve assessing host humoral immunity, detection of fungal antigens using immunologic agents, and detecting the presence of nucleic acids specific to *C. immitis* (Yeo 2002). Other efforts focus on mapping the proteome of different body fluids (in this case bronchoalveolar lavage) in order to assess a “healthy state” and a “disease state” associated with each fluid proteome. If the proteome associated with healthy lung fluid is discovered, it could be used to detect foreign protein, including those associated with *C. immitis*. This method is particularly desirable, as it would be associated with low cost and low level of invasiveness (Hu 2006).

The lack of consistently effective diagnosis and the potential severity of infection lead to the conclusion that preventative measures are the best solution against coccidioidomycosis. Although the development of a consistent, reliable diagnostic test of coccidioidomycosis is critical to the early detection of infection and subsequent treatment, effective vaccination would lower the likelihood of developing primary infection. Vaccination would decrease the money spent on treatment as well as the suffering involved in diagnosis of a severe infection. With the overall increase in the number of cases, the occurrence of cases in non-endemic areas, and growing immunocompromised population, the need for an effective vaccine is apparent and justifiable (Galgiani 2000).

Section 2 – Vaccine Design and Protective Immunity

As a dimorphic fungus, *C. immitis* presents unique complications in terms of antigen selection for vaccination. The fungus can exist in the body both in the infectious arthroconidia form, as well as the parasitic spherule form, inducing different immune responses at different life cycle stages, as previously discussed. Only arthroconidia cause primary infection. Thus, it would be logical to assume that vaccination with arthroconidia-derived antigens would be the most protective. However, animal studies have demonstrated that this is not the case. Instead, it appears that antigens derived from *C. immitis* spherules provide the most protective memory immune response against challenge with arthroconidia. As previously suggested, this may be due in part to the quantity of antigen present on the spherule, rather than a difference in antigen type in the spherule form.

While a number of protein antigens have been isolated and identified as potentially effective vaccine candidates, three have been identified for the purpose of this proposal as providing the most complete protection. Protein antigens Ag2/PRA, CSA, and Pmp1 have been found to induce both Th1 and B cell immune responses and immunological memory. Ag2/PRA, was originally isolated from an extract of the *C. immitis* mycelia. CSA is an exoantigen isolated from filtrates of both mycelia and spherule cultures. Pmp1 is a protein primarily found in the spherule form of the fungus (Cox and Magee 2004) (Osborn et al. 2006).

It has been shown that nearly all of the *C. immitis* protein antigens are associated with induction of hypersensitivity and tenderness at the site of injection. To minimize these negative side effects, recombinant versions of these peptides can be produced, and have shown to be less irritating in animal trials. The production of recombinant protein antigens involves transfecting the protein gene into a vector, such as a bacterial plasmid, which causes the bacteria to produce the desired protein. The protein can then be isolated and purified through extraction. This may also reduce toxicity of unintentional byproducts in extracts. The purified forms of the antigens will provide for a safer, more effective immune response.

In order to properly stimulate an effective memory-inducing immune response, vaccine contents must facilitate immune recognition of antigen. In the case of a multivalent recombinant protein vaccine, naked proteins are not necessarily enough to be immunogenic as they dissipate rapidly in the blood stream, and may not be engulfed or recognized by antigen presenting cells (APC). Use of an adjuvant could solve this problem. Adjuvants provide two imperative functions in a vaccine. First, they ensure the slow release of antigen so the immune system has a chance to recognize the presence of foreign material. Second, adjuvants can actually assist in APC uptake of antigen.

An ideal adjuvant option is Immunostimulatory Sequences with synthetic Oligodeoxynucleotides (ISS-ODNs) combined with antigenic proteins in Immunostimulatory Complexes (ISCOMs) (Joseph 2002). As previously discussed, cell-mediated immunity provides the most effective natural response against *C. immitis* infection. ISS-ODNs contain unmethylated bacterial CpGs, which have been shown to preferentially stimulate cytokines associated with Th1 response, via the MAPK signaling

pathway, including IL-2, IL6, IL-12, INF- γ and TNF- α . Expression of IL-2 and IL-12 in combination directly activates the growth, proliferation, and activation of Th1 and Tc cells (Joseph 2002). Production of IFN- γ increases MHC expression on macrophages for antigen presentation. The ability of ISS-ODN to stimulate cytokine production covers the need for effective presentation of antigen.

The slow-release function of the adjuvant, as well as facilitation of APC-antigen uptake is provided by ISCOMS. Liposomal ISCOMs can serve as effective vehicles for vaccination because liposomes containing antigen and ISS-ODNs can fuse with host immune-cell membranes and promote vaccine delivery through endocytosis. Cellular processing of liposomal contents will lead to antigen presentation and cytokine production which will stimulate immunity (Joseph 2002).

The virulence mechanisms of *C. immitis* allow it to effectively evade the immune response both intra- and extracellularly. A significant aspect of this is the modulation of the immune response towards a Th2-biased state. Th2 helper cells drive B cell differentiation and the production of antigen-specific immunoglobulins, which should constitute part of a balanced immunological response to any pathogen, including *C. immitis*. However, high titers of antibody in patients with coccidioidomycosis have been correlated with extra-pulmonary dissemination of the disease. *C. immitis* can coordinate the production and shedding of SOW material in order to stimulate a humoral immune response that is ineffective against the growing spherules, often too large to be phagocytosed. This allows the released endospores, seemingly devoid of the antigenic components targeted by the humoral response, to escape and continue the parasitic cycle. Even when phagocytosed, spores can avoid destruction inside macrophages by impinging and even manipulating key processes such as the respiratory burst. All these issues indicate that a robust, Th1-mediated cellular immune response is extremely vital to counter an infection by *C. immitis*. This need is exemplified in the immunocompromised whose scarce numbers of CD4 lymphocytes render them vulnerable to a disseminated infection by *C. immitis* and other opportunistic pathogens.

A vaccine against *C. immitis* will aim to prime the cell-mediated immune response by delivering key antigens to APC. The specific antigens chosen have been exhaustively studied and found to be competently immunogenic. Multivalent recombinant peptide vaccines, as mentioned previously, have been tested in murine models yielding impressive results. Upon vaccination, tissue dendritic cells and macrophages, especially in the alveolar compartments, will uptake and process the chosen recombinant peptides. The ISCOM adjuvant will directly deliver the recombinant peptides to the MHC I processing for the activation of naïve CD8 lymphocytes. The introduction of ISS-ODN will also stimulate the NF κ B and MAPK signaling pathways in antigen presenting cells leading to the production of cytokines involved in cellular immunity, such as IL-6, IL-12, IL-18 and IFN- γ . IFN- γ is especially important in promoting CD4 lymphocyte differentiation into the Th1 subset and inhibiting Th2 cell growth.

This vaccine will activate CD4 and CD8 lymphocytes in order to suppress the parasitic cycle of *C. immitis* and clear an infection. Cytotoxic T cells that have been activated by

MHC I will target infected cells in order to eliminate spores attempting to evade the phagocytic effectors of innate and adaptive immunity. Although tissue damage may occur, this may also prevent the rapid spread of spores across the peripheral tissues. Influenza-like symptoms elicited in a patient immunized by our vaccine after encountering *C. immitis* may signal that the arthroconidia are being contained in the mucosal epithelia by the inflammatory response and cellular cytotoxicity. Cytotoxic cells will release granzymes and induce apoptosis through Fas ligand binding to infected cell Fas receptor. However, the most important aspect of this vaccine is the activation of Th1 helper cells. Cytotoxic cells will be effective against spores that are present inside host cells that have not yet entered the parasitic cycle. Primed Th1 cells will activate macrophages, through CD40 and cytokines, to kill ingested spores very early on during the infection. First, this will ensure that spherules are phagocytosed before they grow too large and attempt to modulate the immune response. Second, macrophage activation will promote phagosome-lysosome fusion and respiratory burst before ingested spores can counter these mechanisms. In addition, effective co-stimulation of CD8 T cells by antigen presenting cells may only occur in the presence of a Th1 cell line that is further stimulating the latter.

A balanced cellular and humoral response is needed to fully contain *C. immitis* and eliminate it from the body. Naïve B cells may encounter *C. immitis* or the recombinant peptides and present antigen on MHC II and activate the Th2 subset. After cellular immunity has been provoked and is active in the mucosa and other tissues, the Th2 subset will signal B cells, especially through IL-4 and CD40 ligand, to proliferate and produce IgA and IgG antibody that can reach the tissues and supplement adaptive immunity. IgA already present in the mucosal areas and binding to spores will trigger respiratory burst in neutrophils and other inflammatory responses in cells expressing the Fc receptor for IgA. The secretion of IgA and IgG, although late compared with the cellular response, will confer protection to most tissues by targeting any spores that escaped the mucosal areas and inevitably alert the already vigorous Th1 and inflammatory responses as well as clear any debris produced by cytotoxicity and pathogen degradation. APC not only activate the effector functions of naïve T lymphocytes but also drive their differentiation and proliferation, especially by inducing production of IL-2. The production of memory T cells during proliferation will ensure a swift and effective response against future infection by *C. immitis* by an early sustained cellular response and later humoral activity.

As with any vaccine, target populations must be carefully considered. Because the control of *C. immitis* is dependent on a cellular immune response, and because this vaccine seeks to target Th1 and Tc immune responses specifically, individuals with immune deficiencies related to cellular immunity would not be good candidates to receive this vaccine. Unfortunately, this vaccine does not address protection of this population. Further study of the B-cell function in controlling initial infection would be necessary in order to tailor a vaccine to these individuals. In addition, individuals already infected with *C. immitis* even if asymptomatic, would have no need for vaccination. Thus, the most logical candidates for vaccination would be individuals who are currently immunologically negative for *C. immitis*, who may find themselves in high risk situations, including people relocating to endemic areas from non-endemic areas,

individuals in endemic areas who expect higher exposure to the pathogen, like those who work outdoors, especially in occupations involving soil disturbance.

In total, the proposed antigen makeup for this vaccine will include recombinant Ag2/PRA (rAg2/PRA), rCSA, and rPmp1. It will be administered subcutaneously in combination with an ISS-ODN adjuvant in an ISCOM vehicle. The target population will be individuals currently negative for *C. immitis* specific antibody, who will be in situations of high risk of exposure and infection, as described above.

Section 3 – Vaccine Efficacy

The *C. immitis* vaccine will be administered as an injection subcutaneously, rather than intramuscularly, in order to reduce pain at the site of injection. Oral vaccination is not a viable option for protein antigens, as they will be digested. Intranasal vaccination, though an option, can incur other side effect, and primarily stimulates undesired humoral mucosal immunity. A multivalent recombinant protein vaccine is efficient because there is not excess material and therefore the subunits do not stimulate inflammation. The vaccine should elicit high response levels of Th1 and lower response levels of antigen-specific IgA and IgG. The vaccine will be administered at the physician's discretion depending on their patient's age, health and immune competency.

Trials:

Control: 50 volunteers from an endemic area who test negative for spherulin and coccidioidin antibody, given a placebo vaccine.

Test group: 50 volunteers from an endemic area who test negative for spherulin and coccidioidin antibody, given the actual vaccine.

Measuring immunity

Two weeks after vaccination, T and B cell responses should be at a peak of activity. At this point, blood will be drawn from all test participants, and tested for both T and B cell response, as described below.

Measuring T cell-mediated immunity

The aim of this vaccine is to stimulate vigorous T cell-mediated immunity and limit the spread of coccidioides infection. Activated Th1 and cytotoxic T cells will circulate from the peripheral lymphoid organs, where they will be activated, through the bloodstream and to the site of infection. In order to assess the effectiveness of our vaccine we must detect the level of T cell-mediated immunity elicited after immunization. To do this we must isolate the specific lymphocyte subsets that would be produced in order to mediate the expected cellular immune response, namely CD8 and CD4 lymphocytes. Although cytotoxicity is important in cell-mediated immunity, detection of differentiated T-cell function through secreted cytokines is of primary importance.

The first step in a T cell evaluation scheme involves the isolation of lymphocytes from the peripheral blood by density centrifugation. Of course, the principal events in T cell activation and effector function would occur on lymphoid organs, mucosal and mucosal-

associated lymphoid tissues. Extraction of lymphocytes from these sites in humans would be painful and laborious and would deter participation in our clinical trials. Collection of sputum from the respiratory tract is also a less attractive idea as few lymphocytes would be obtained. Therefore activated circulating lymphocytes recovered from patient blood over the course of trial will be used.

The most powerful tool to classify and enumerate isolated lymphocyte -population is flow cytometry and with a fluorescence-activated cell separator (FACS). After separating T from B lymphocytes by antibodies targeting the constant regions of B- and T-cell antigen receptors, we must subdivide the T cell population on the basis of co-receptor expression. Next we want to separate the CD4 and CD8 cells (as with separating T and B lymphocytes) by FACS, with differently labeled antibodies specific for each co-receptor. The active effector functions of the subsets will also be characterized, after presenting them with our vaccine antigens, mainly by cytokine secretion. To analyze cytokine secretion we will employ the cytokine capture method where we will use a hybrid antibody constructed from antibodies specific for a particular cytokine and a common cell-surface protein of a given lymphocyte. A fluoro-chrome-labeled secondary antibody specific for a different epitope of the chosen cytokine will allow detection by flow cytometry. The advantage of this method is that the lymphocytes will not be destroyed and will exhibit how profusely they actually secrete cytokines.

A principal concern is with the activity of Th1 cells and macrophage activation. Therefore, hybrid antibodies specific for CD4/IFN- γ will detect specific cytokine secretion. In addition to their cytotoxic functions, activated CD8 T cells may also release IFN- γ and other cytokines to promote antiviral activity in infected cells. Thus CD8/ IFN- γ hybrid antibody can be used to detect CD8 IFN- γ release. For the CD4 subset, fluoro-chrome-labeled antibody specific for the CD40 ligand will be added, since together with IFN- γ secretion these two signals are the hallmarks of macrophage activation by Th1 cells. Th2 cells also use CD40 ligand to activate B cells to produce antigen-specific antibody and so measuring the cytokine and ligand parameters would allow the two subsets to be identified. This assessment can help cement the precise response evoked by our vaccine. Cytotoxicity assays can also be carried out in addition to IFN- γ secretion by CD8 cells using a chromium release cytotoxic assay.

Test to measure B cell memory

After vaccination, one could test for the presence of B cell memory by testing for B cell-secreted IgA and IgG. Presence of cocci-specific IgG in the blood can be verified via an ELISA test. One could fix a plate with cocci antigen and wash it with serum from a vaccinated person. Since B cell memory takes a few weeks to be activated, completion of this assay must be delayed enough time to allow the formation of specific B cells. After vaccination and an appropriate stimulation period, serum from a vaccinated individual can be used to wash a plate fixed with Cocci antigen. The next step would be to wash the plate of unbound elements and then apply an anti-IgG antibody with an enzyme attached to the Fc portion. The anti-IgG antibody would bind to the specific antibody and stick. The plate could then be washed again and undergo an application of the enzyme's

substrate. If a measurable reaction occurs, this indicates the presence of secreted Ig specific to *Coccidioides* antigen. Secreted IgG is a good indicator that memory B cells has differentiated within the system. An ELISA test for mucosal IgA could also be performed, but it would be redundant.

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