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Antibody enhanced intracellular killing of Leishmania amazonensis: the role of soluble immune complexes and their effect on autophagy

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Antibody enhanced intracellular killing of *Leishmania amazonensis*: the role of soluble immune complexes and their effect on autophagy

by

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ABSTRACT

Leishmania amazonensis is an intracellular protozoal parasite that causes cutaneous leishmaniasis in humans and other mammalian hosts. This disease affects people within tropical and subtropical countries. Generally a Th1 cell-mediated host immune response is thought to be important for the clearance of the parasite. However, throughout this work we have shown that a productive B cell response is important for the clearance of the parasite through the production of IgG2a isotype antibodies. These antibodies can form small soluble immune complexes that can stimulate the FcγR leading to the production of superoxide. Superoxide and nitric oxide are required to kill intracellular *L. amazonenesis* parasites. Our studies have also shown that macrophages can be activated to produce these required immune factors if they are stimulated with soluble immune complexes, IFN-γ, and *Leishmania* antigen (tripartite activation). We have also found that these three factors can lead to the upregulation of the autophagy pathway for the clearance of the parasite. These soluble immune complexes can be replaced by novel recombinant proteins that have similar morphology to murine IgG2a Fc. These Fc constructs have the ability to recapitulate killing of the parasite and superoxide production seen with tripartite activation. All of these factors are important for the development of a possible immunomodulating therapy that could be used to treat patients infected with this chronic, debilitating disease.

CHAPTER 1

GENERAL INTRODUCTION

Leishmaniasis – Introduction, History, Epidemiology:

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the *Leishmania* genus (*Kinetoplastida: Trypanosomatidae)*. Leishmaniasis has been present in the human population as far back as humans were able to document its presence. *Leishmania* mitochondrial DNA has been isolated from mummies from Egypt and Sudan as early as 1500 BC and from America as early as 800 BC, before the arrival of Europeans in America.[1] In 1903, *Leishmania* was named after Dr. William Boog Leishman found characteristic bodies in the spleen of infected patients.[2] Leishmaniasis also has several other names including: leishmanasis, leishmaniose, Oriental boils, Baghdad Boil, kala azar, black fever, sand fly disease, Dum-Dum fever, or espundia.[3]

There are 30 different *Leishmania* species, approximately 20 of which have the potential to infect humans. Human infections are zoonotic; the result of a recent host transfer from a zoonotic source; or as occurs in urban areas, are anthroponotic. These protozoan parasites are transferred to the human by the bite of infected female phlebotomine sand flies.[4] There are three main forms of the disease, including cutaneous, visceral, and mucocutaneous. These different forms of disease are caused by different *Leishmania* species.

Leishmania species are divided into Old World and New World regarding their geographic location. The Old World (Eastern hemisphere) leishmaniasis include *L. donovani, L. infantum, and L. major*. The New World (Western

hemisphere) leishmaniases include *L. chagasi* (*L. infantum*), *L. braziliensis* and *L. amazonensis.*

Leishmaniasis occurs in more than 90 countries in the tropics, subtropics, and southern Europe.[5] There are approximately 310 million people at risk of infection with approximately 1 million cases of cutaneous leishmaniasis reported in the last five years and 300,000 cases of visceral leishmaniasis with more than 20,000 deaths annually.[4] Over 90% of visceral leishmaniases occur in six countries, including Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan. Most cases of cutaneous leishmaniasis occur in Afghanistan, Algeria, Brazil, Columbia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia, and the Syrian Arab Republic.

Transmission

Leishmaniasis is currently classified as a neglected tropical disease. *Leishmania sp*. are mainly transmitted by phlebotomine sand fly vectors between humans and other mammalian hosts, making transmission of the disease very difficult to control. Rarely within urban areas there is anthroponotic transmission of cutaneous and visceral leishmaniasis.

L. amazonensis causes cutaneous leishmaniasis in the New World. Although it is not considered a frequent cause of New World cutaneous leishmaniasis, less than 3% of cases, it is a unique species that can lead to severe disease with an outcome of diffuse cutaneous leishmaniasis. *Leishmania amazonensis* is transmitted by sand flies that take a bloodmeal from an infected host and inject the infective metacyclic promastigotes into the skin of a human or

other mammal. The promastigotes then transform into amastigotes and multiply inside macrophages where another sand fly can later take up the infected cells, which transform from amastigotes to promastigotes within the midgut of the sand fly.[5] (Figure 1)

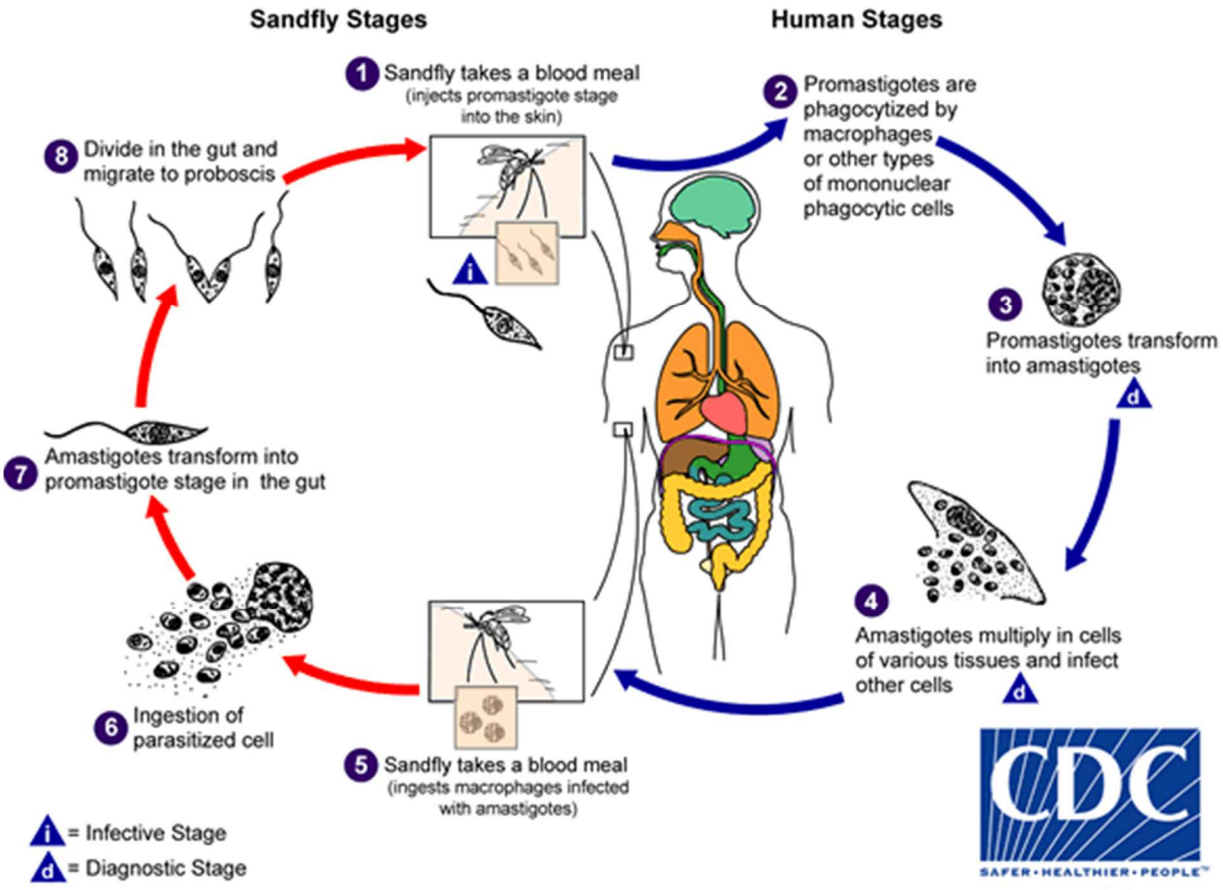

Figure 1. Life cycle of *Leishmania,* www.cdc.gov, accessed October 20, 2015. Leishmaniasis is transmitted by the bite of a sand fly. The sand fly takes a blood meal and injects the promastigote into the bloodstream. The promastigote is taken up by macrophages and transforms into amastigotes. Amastigotes replicate and reside within macrophages where they can be taken up by sand flies when they take a blood meal. In sand flies, amastigotes transform back to promastigotes within the sand fly hindgut.

Disease, Diagnosis, Treatment, Prognosis

There are three main diseases in humans including: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis. These three different forms of disease are caused by different species of *Leishmania* and the different host response to the parasite.

Visceral leishmaniasis is the most severe form of disease. It is caused by *L. donovani* and *L. infantum*. The clinical symptoms associated with VL include fever, anemia, splenomegaly, hepatomegaly, and progressive cachexia. This disease occurs most frequently in immunosuppressed individuals, such as HIV infected patients. Other symptoms can include lymphadenopathy and persistent diarrhea. The prognosis of this disease is varied and spontaneous recovery can occur; although, full-blown disease (untreated) is fatal.[6]

Cutaneous leishmaniasis is caused by *L. major* (Old World) or *L. amazonensis* and *L. braziliensis* (New World), along with many other species. The lesion associated with CL typically first appears as a persistent insect bite that gradually enlarges. The lesion can then become an open sore. Spontaneous resolution generally occurs, however the time for resolution varies depending on the species of the parasite and host response.[6] Diffuse cutaneous leishmaniasis (DCL) is a manifestation of cutaneous leishmaniasis in which lesions may be restricted or widespread over the body and can be caused by *L. amazonensis*. DCL is generally restricted to Venezuela and the Dominican Republic in the western hemisphere. DCL occurs due to the lack of an effective host immune response and there are typically numerous parasites within lesions. [6]

Mucocutaneous leishmaniasis occasionally occurs and is caused by *L. braziliensis (*New World). Mucocutaneous leishmaniasis occurs at or close to a mucosal surface after the bite of a sand fly. Classic mucocutaneous leishmaniasis occurs following the resolution of a cutaneous lesion. The clinical symptoms associated with this disease include destruction of mucosa and associated cartilage and the disfiguration of the face or affected surface.[6]

Diagnosis of leishmaniasis can be difficult to achieve. It occurs mainly by clinical exam in endemic areas; however confirmation of the disease diagnosis can be established through the demonstration of amastigotes in infected tissues. This can be achieved by fine needle aspirates or tissue biopsies of bone marrow and spleen. Other assays have been developed for the diagnosis of leishmaniasis, including serology and PCR. Serologic assays are most commonly used for diagnosing visceral leishmaniasis and these assays include antigen-based direct agglutination tests and commercially available immunochromatographic dipstick tests. These tests have high sensitivity and specificity.[7] Another assay that is occasionally used for the diagnosis of cutaneous leishmaniasis is the Montenegro skin test (MST). This test has high sensitivity and specificity, however it does not distinguish between past and present infections.[7] PCR can also be used for the detection of *Leishmania* nucleic acids and allows for a very specific and sensitive result. This method can be used for the quantification of parasite load following treatment or to identify the *Leishmania* species causing the infection.[7] In a clinical setting a combination

of these different diagnostic assays would be used to help confirm a diagnosis of leishmaniasis.

There is currently no vaccine available for human leishmaniasis. Visceral leishmaniasis can be difficult to treat and is often fatal in full-blown infections. The traditional treatment of VL has been the use of pentavalent antimonials, however other treatments have also been developed. These treatments include amphotericin B, Paromomycin, and miltefosine. These drugs have both advantages and disadvantages, and none of them are 100% effective in parasite clearance. Several drugs have been implicated for oral treatment; some examples include allopurinol, azole drugs and miltefosine. The allopurinol and azole drugs have little evidence or scientific research to show clinical efficacy, therefore their use is not recommended.[8] Miltefosine is another oral drug that has demonstrated good clinical efficacy of cure rates at approximately 85%. However, relapse 12 months following miltefosine treatment can be as high as 20%.

Parenteral treatments for leishmaniasis are also available and these include pentavalent antimonials, Amphotericin B deoxycholate, liposomal Amphoteracin B, Pentamidine, and Paromomycin. Pentavalent antimonials were considered the first line of treatment and have been used since the late 1940s although parasite resistance has development in certain geographic areas. Parasite resistance is particularly established in India where resistance rates are as high as 60%.[8] Cure rates with pentavalent antimonials range from 80-100%. Cardiac toxicity with pentavalent antimonials can reach as high as 17% if

treatment regimens extend longer than 28 days.[8] Amphotericin B deoxycholate and liposomal Amphotericin B are other first line drugs against leishmaniasis. These drugs are very effective and can have cure rates ranging from 95-100%, with little toxicity. Paromomycin is not recommended as a sole therapy against VL as cure rates were as low as <50% in one study.[8]

These drugs have high rates of toxicity and variable efficacy. All of these treatments can have different beneficial aspects that allow for their preferential selection or a combination of different treatments within patients. Other factors also need to be considered when treating a patient, such as the development of parasite drug resistance, HIV-*Leishmania* co-infection, malnourishment, or other co-infections.

Many cases of cutaneous leishmaniasis will spontaneously resolve regardless of the treatment. Cutaneous and mucocutaneous leishmaniasis treatments are often poorly justified and have limited effectiveness. Nevertheless, many treatment options are available including physical therapies, topical drug therapies, oral drug therapies, and parenteral drug therapies. The treatment chosen for patients with cutaneous and mucocutaneous leishmaniasis greatly depends on the species of *Leishmania* responsible for disease. Old World leishmaniases are more likely to spontaneously resolve than New World leishmaniases or mucocutaneous disease. These facts lead most physicians to treat Old World cutaneous lesions with physical therapies such as thermotherapy, cryotherapy, or CO_2 laser treatment. For New World cutaneous leishmaniasis the preferred treatment is systemic therapy, however this includes

risky side effects and long treatment periods. In the case of cutaneous leishmaniasis caused by *L. amazonensis* and mucocutaneous leishmaniasis, pentavalent antimonials seem to be the best treatment option.[9]

More research is needed to develop better treatment options with less severe side effects and better efficacy. These treatments should also lead to quicker lesion resolution and prevent disfiguration.

Murine cutaneous Leishmaniasis

The mouse model is a very important tool for the study of the host immune response to *Leishmania* parasites. Both *L. major* and *L. amazonensis* cause cutaneous leishmaniasis in mice after experimental infection. Genetically susceptible and resistant strains of mice have been pivotal in understanding the immunopathogenesis of leishmaniasis. Depending on the mouse strain and the species of *Leishmania* some animals develop a lesion that heals after a variable amount of time while others may develop progressive lesions that eventually lead to the death of the animal.[10] BALB/c mice develop uncontrolled disease when infected with either *L. major* or *L. amazonensis* and other mouse strains show various levels of susceptibility to these pathogens. C3H mice are able to heal infection with *L. major* but develop chronic disease when infected with *L. amazonesis*. C57BL/6 and C57BL/10 mice also heal *L. major* infections but develop chronic lesions that can metastasize when infected with *L. amazonensis*.[10] The genetic predisposition for susceptibility to *L. major* infection in mice is determined by their dominance of a Th1 or Th2 phenotype driven by IFN-γ and IL-12 or IL-4 respectively.[11]

C3H mice that are coinfected with *L. major* and *L. amazonensis* or prior infection with *L. major* can confer protection against *L. amazonesis.*[12] The outcome of the host immune response to *Leishmania spp.* are dependent upon the host immune response, nutritional status, and parasite species or strain.[13] Infection of mice with *Leishmania major* can lead to cutaneous leishmaniasis and healing of that infection requires the production of Th1 cytokines, the activation of macrophages, and the production of reactive oxygen species and reactive nitrogen intermediates, such as nitric oxide.[14, 15] Alternatively, infection of mouse models with *L. amazonensis* can lead to non-healing cutaneous leishmaniasis, as these parasites are highly resistant to many neutrophil and macrophage derived leishmanicidial effector molecules.[14, 15] The *Leishmania* immunology paradigm has been well described using the BALB/c mouse model and *L. major* infection. However, there are many complexities in the mechanisms responsible for immunity against these pathogens, along with significant species variation, therefore research is ongoing.

L. amazonensis is more difficult to eliminate and causes chronic infections in comparison to *L. major*. Our lab has shown that an impaired immune response to *L. amazonensis* may be a result of an impaired ability of immune cells to transition from a naïve to an effector phenotype. *L. amazonensis* infected dendritic cells have altered expression of surface markers, cytokine production, maturation, and function. These include significantly reduced CD40 surface expression and a decreased number of IL-12p40 producing cells within the draining lymph node of *L. amazonensis* infected mice.[16] $CD40L^{-/-}$ are more

susceptible to *L. amazonensis* infection, suggesting the CD40-CD40L interactions are important for the generation of a host cellular immune response.[17] Lipophosphoglycan (LPG) a glycolipid on the surface of *Leishmania* has been shown to activate extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), and the p38 MAP kinase, and both p38 and ERK appear necessary to induce IL-12 and nitric oxide production. Inhibiting the phosphorylation of ERK in vitro can result in recovering the DC phenotype resulting in an increase in IL-12 production.[16] Some other parasite virulence factors include the modulation of TLR signaling, IFN-γR downregulation, and the induction of IL-10.[18] All of these responses skew the host immune response to favor parasite survival. LPG on *L. amazonensis* can also affect host cell kinases and phosphatases. LPG can interfere with the binding of regulators such as Ca^{2+} and can block protein kinase C (PKC) membrane insertion and activation.[19] The blockage of PKC can have a critical role in preventing superoxide production through the downstream effects of nicotinamide adenine dinucleotide phosphate (NADPH) complex assembly.[19] There are multiple virulence factors that *Leishmania* uses to prevent a protective host immune response and to favor the intracellular survival and replication of amastigotes. As these parasite induced virulence factors are elucidated, the modulation of these specific signaling pathways may be effective in immunomodulatory treatments. This has the potential to lead to a decrease in intracellular parasite survival and replication.

The role of B cells in Leishmaniasis

B cells are generally not given much attention in *Leishmania* infections, as the focus remains on the generation of a productive Th1 response with IFN-γ and IL-12 for lesion resolution. However, our lab has shown that B cells may be playing a larger role in the host's immune response to *Leishmania* than previously thought. Our lab has demonstrated that CD4+ T cells and B cells can limit *L. amazonensis* survival in macrophages in vitro, and this correlates with susceptibility to *L. amazonensis* infection by C57BL/6 mice, which have a deficiency in their B cell response.[20, 21]

B cells develop from a single precursor bone marrow stem cell line and mature into many mature B-cell subsets, including memory B cells and plasma cells. B cell subpopulations are distinguished by their receptors and cell surface markers of activation or differentiation. The different B cell subsets include pro-B cells, pre-B cells, immature and transitional B cells, plasmablasts, and plasma cells.[22]

B cells can be found throughout the body in a number of organs in a normal individual. These include in the bone marrow (precursors, plasma cells), blood (transitional, naïve mature, and memory B cells, plasmablasts, plasma cells), secondary lymphoid tissues (mature naïve, memory B cells). B cells subpopulations display many similar cell surface markers although some differ amongst populations. One example is the presence of CD27 as a marker of memory B cell populations. CD20 antigen is expressed on a majority of B cells, but not stem cells, pro-B cells, or terminally differentiated plasma cells.[22] CD20

antigen is a transmembrane protein that is thought to be involved in the activation and proliferation of B cells.

Described disorders of the B cell lineage mainly consist of neoplastic or autoimmune disorders. B cell lymphomas occur in humans and animals and consist mainly of follicular lymphoma, non-Hodgkin's lymphoma, and chronic lymphocytic leukemia.[22] Numerous autoimmune disorders are described throughout the literature. These diseases can occur through the production of autoantibodies by B cells or plasma cells. Some examples include rheumatoid arthritis, granulomatosis with polyangitis (GPA), and microscopic polyangitis (MPA). The use of antibodies against B cells was developed in the late 1980s and 1990s, where an antibody is used to deplete the specific cell type by antibody dependent cell-mediated cytotoxicity. Rituximab, an anti-CD20 monoclonal antibody (Rituxan ©, Genentech, Biogen, IDEC) was first licensed for use in 1997 against follicular lymphoma and is now licensed for use against Non-Hodgkin's Lymphoma (NHL), Chronic Lymphocytic Leukemia (CLL), Rheumatoid Arthritis (RA), Granulomatosis with Polyangitis (GPA) (Wegener's Granulomatosis), and Microscopic Polyangitis (MPA). Since that time other anti-CD20 antibodies have been developed for use and include Ofatumumab, Ocrelizumab, Veltuzumab, AME-133v, PRO131921, or GA101.[23]

Anti-CD20 therapy has been successful in the depletion of B cells in humans and mice with very few reported side effects. Different subpopulations of B cells along with the location of the B cell population yield different responses to the anti-CD20 therapy. B cells within the peripheral blood are most susceptible to

anti-CD20 depletion therapy and many studies report a depletion efficiency of 99% or higher.[22] Populations that are more resistant to therapy include peritoneal B1-type B cells, germinal center B cells, and marginal zone B cells.[22, 24] These differing sensitivities may be due to the inefficiency of effector cells at getting to these locations or to a defect in antibody-dependent cellular cytotoxicity. For example, the FcR is important in B cell depletion and polymorphisms of this receptor can effect the efficiency of anti-CD20 therapy.[25] Some suggest that higher doses of antibodies are needed to deplete B cell populations in bone marrow, spleen, and lymph nodes in successive order.[22] Depletion of solid organs is often not complete.

Despite relatively high depletion of B cells following anti-CD20 therapy there are relatively few studies demonstrating an increased susceptibility to infections. Patients receiving rituximab therapy will be immunosuppressed and may be more susceptible to chronic infections such as leishmaniasis. Many clinical trials have conflicting results regarding the association of anti-CD20 therapy and infections. One study has shown an increased incidence of infections of patients with lymphoma and rheumatoid arthritis receiving rituximab.[26] A review of recent data showed that rituximab therapy significantly increased the risk of infection in patients with lymphoma or other hematological malignancies; however, this increased risk of infection was comparable to other concurrent treatments in patients with rheumatoid arthritis.[27] Patients receiving rituximab therapy will be immunosuppressed and may be more susceptible to chronic infections such as leishmaniasis.

Patients receiving B cell depletion therapy will repopulate their B cells within 6 to 9 months following therapy. This is through the production of naïve B cells from the bone marrow stem cells, which then repopulate the peripheral blood and secondary lymphoid organs. Plasma cells are often long lived and are not affected by anti-CD20 therapy, however the production of new plasmablasts and plasma cells will be delayed.

Macrophages

Macrophages are important immune cells derived from bone marrow monocytes that are integral in bridging innate and acquired immune responses. Macrophages are phagocytic cells that can travel to the lymph node and present antigen to T cells. Macrophages have numerous receptors that allow them to phagocytize foreign antigens. The receptors that are reported to play a role in the uptake of *Leishmania* include the third complement receptor (CR3), first complement receptor (CR1), mannose receptor (MR), Fc gamma receptors (FcγRs, in particular FcγRII), and fibronectin receptors (FnRs).[28]

Macrophages have many roles as a part of the innate immune system. These roles include recognition and phagocytosis of foreign antigens along with different mechanisms employed to ingest those foreign substances. Some ways that macrophages kill phagocytized microorganisms include through the acidic environment of the lysosome, production of nitric oxide, and superoxide. Macrophages follow two main phenotypes, M1 and M2. M1 macrophages are characterized by the production of reactive oxygen and nitrogen species,

whereas M2 macrophages are characterized by their anti-inflammatory and wound repair properties. For the extent of this review we will focus on M1 macrophage phenotype.[29] These macrophages are stimulated by IFN-γ and play an important role in innate immunity.

It is generally accepted that a protective immune response against *Leishmania* is dependent upon the induction of a CD4+ Th1 response.[30] CD4+ Th1 T cells produce IFN-γ that activates macrophages to kill intracellular parasites. IFN-γ induces macrophages to synthesize nitric oxide as well as superoxide. Nitric oxide has been shown to play an important role in antimicrobial functions and has been shown to be a critical molecule in the parasitical function against *Leishmania major* [31] and has been shown to limit growth of *Leishmania* within infected macrophages.[30] Nitric oxide is generated from L-arginine by the enzyme NO synthase (NOS).[32] There are three forms of NOS, including inducible, neuronal, and endothelial. Inducible NOS (iNOS) is induced by the presence of cytokines activating neutrophils, macrophages, and other white blood cells.[33]

The production of reactive oxygen species has also been shown to play an important role in the clearance of *Leishmania amazonensis.*[30] The production of ROIs is initiated by NADPH oxidase. NADPH oxidase is activated when the cytosolic proteins (gp40phox, gp47phox, gp67phox, and Rac2) translocate to and interact with the membrane bound proteins (gp91phox, gp22phox, Rap1a) to make the active NADPH oxidase. NADPH oxidase produces superoxide, which is

used to control intracellular microbes.[34] NADPH oxidase can be activated by IFN-γ, IL-8, or by IgG binding to Fc-receptors or microbial products (such as LPS).[34]

Leishmania exhibits many virulence factors that allow it to modulate the host immune response in order to ensure its survival. These include modulating receptor responsiveness in macrophages and altering host cell kinases and phosphatases. Some receptors on the surface of the macrophage that are affected by *Leishmania* include CD40, TLRs, IFN-γ, and IL-10 receptors. Several studies have shown that *Leishmania*-infected macrophages have MAPKs that are repressed, causing a decrease in IL-12 and $iNOS_2$ production.[35] Parasite LPG has been implicated in the inactivation of MAPKs.[35] Our lab has also shown that *L. amazonensis* amastigotes inhibited the ability of DCs to undergo proper maturation.[16] TLRs are thought to be modulated by *Leishmania* either through the suppression of TLR 2, 4, 9, or the adaptor protein MyD88. TLR 4 deficient mice had impaired resistance to *L. major* and TLR9 signaling is suppressed by *L. major* infection. The modulation of TLRs or other pattern recognition receptors would lead to a decrease in the cytokines (such as IL-12) produced along with a decrease in the induction of iNOS expression.[35] *Leishmania* can also inhibit IFN-γ receptor signaling through the inhibition of the JAK2/STAT1 pathway. The suppression of IFN-γ receptor pathways leads to a decrease in nitric oxide production and a decrease in MHC class II expression. Finally, host IgG present on the surface of *Leishmania* amastigotes can interact with the FcγR on the surface of the macrophage leading to the induction of IL-10, a potent immunosuppressant.[19]

Leishmania LPG has been shown to inhibit protein kinase C activation and subsequent intracellular signaling. *Leishmania* also activate phosphoinositide-3-kinase (PI3K) signaling, which has been shown to negatively regulate IL-12 production. In conclusion, there are many ways that *Leishmania* has evolved to use the host cell molecules to its advantage to allow for its intracellular survival.

Fcγ Receptors

Antibody Fc (Fragment crystallizable) receptors, found on many cell surfaces, that mediates binding of immunoglobulin, can induce a variety of immune functions by regulating intracellular signaling. Antibody Fc receptors (FcR) are named by their ability to bind specific immunoglobulin subtypes. FcγRI (CD64) resides on the surface of monocytes and macrophages, and binds the Fc portion of IgG1 or IgG2a in humans or mice, respectively, with high affinity. The main immune functions of Fc receptor engagement are facilitation of phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), induction of the release of inflammatory mediators, and the regulation of lymphocyte proliferation and differentiation.[36] Given their wide array of functions in modulating the immune response, new and promising strategies are unfolding for developing molecules that are able to mimic the FcR-Ig interaction to exploit immunoregulation.[37]

Cell surface expression of FcγRI can be induced by IFNγ. FcγRI can bind monomeric IgG and take it up via endocytosis, however this generally leads to a recycling pathway unless the receptor is cross-linked with multivalent IgG complexes.[38] Cross-linked receptors lead to internalization of the antigen-

antibody complex and activation of the cell to produce effector functions, such as superoxide production.[39] Phagocytosis of the antigen-antibody complex by macrophages results in a pro-inflammatory response and the production of superoxide within the phagosome.[40] However, the size of the immune complex (IC) binding FcγRI affects the signaling pathway that ensues.[40] FcγRI signals through a common γ chain and activates the proto-oncogene tyrosine-protein kinase (SRC) family of kinases, spleen tyrosine kinase (SYK), and has been shown to induce superoxide production when bound by large insoluble IC.[41] However, when small soluble ICs bind the FcγRI they are internalized by endocytic pathways, leading to other functions, such as receptor expression, regulation of signal transduction, antigen presentation, and recycling of those receptors.[42] Other studies have shown that these small soluble ICs can also induce superoxide production upon cross-linking of the FcγRI.[43, 44] Further work is needed to elucidate how clustering of this single receptor can lead to different immune functions at the molecular level. The ability to manipulate these receptors in order to get a timely and localized release of inflammatory mediators can be helpful in inducing an effective immune response against intracellular pathogens.[43, 44]

Autophagy

Autophagy is an intracellular homeostatic mechanism important for the degradation of cytosolic components that range in size from single proteins to entire organelles via autodigestion through the lysosomal pathway. Multiple

autophagic pathways exist in mammalian species. Autophagy plays a role in cell survival throughout health and disease including, aging, cancer, neurodegenerative diseases, immunity, infectious diseases, and chronic inflammatory conditions.[45]

The process of canonical macroautophagy can be broken down into four discrete steps: induction, formation of the autophagosome, autophagosome docking and fusion with the lysosome or vacuole, and autophagic body breakdown.[46] Canonical autophagy can be induced through nutrient deprivation or the inhibition of the phosphorylation of mTOR (mammalian target of rapamycin), such as occurs with Rapamycin.[46] The initiation of autophagy is dependent on the ULK1 complex. Next, a double membrane vesicle begins to form in the cytosol, resulting in the sequestration of cytoplasmic components. This process is highly regulated under the control of GTPases, class III phosphatidylinositol-3-kinases (class III PI3K), and various phosphatases.[46] Fusion of the autophagosome with the lysosome depends on microtubules and vacuolar protein sorting (Vps) protein complex which function to ensure efficient fusion of the autophagosome with the vacuole.[46] Autophagosomes can be recognized by their microtubule associated protein 1A/1B light chain protein 3 (LC3), which is conjugated with phosphotidylethanolamine (PE) to form LC3II. LC3II facilitates the formation of the double-membrane autophagosome; which fuses with a late endosome or lysosome to form the autolysosome.[47] Fusion causes the release of the single-membrane bound inner vesicle of the autophagosome, into the vacuole lumen and the autophagic body is broken

down.[46] Xenophagy is another type of canonical autophagy that targets bacteria for clearance by autophagy.

Non-canonical autophagy occurs without the formation of a double membrane and can lead to autophagosomal degradation through different variations of the autophagy pathway.[48] These pathways are triggered by several cell surface receptors and can be involved in a form of selective autophagy. One pathway is known as LC3-associated phagocytosis (LAP), which links signaling during phagocytosis with recruitment of phagocytosis machinery.[49] The recruitment of LC3II to the phagosome is preceded by class III PI3K activity, similar to canonical autophagy.[50] LAP focuses on extracellular pathogen degradation within a phagosome, whereas canonical autophagy targets bacteria, parasites, and viruses (xenophagy) that either disable the phagosome or that escape into the cytosol.[50] Both non-canonical and canonical autophagy play complementary functions in immunity, and are important in modulating inflammatory pathways.[50] Others have also found that this pathway may occur through other pathways that do not involve PI3K, such as diacylglycerol dependent PKCδ activation.[51]

Autophagy has been shown to play a role in innate immunity and can be activated through numerous pattern recognition receptors to participate in the elimination of microorganisms. Inflammatory cytokines can also be involved in the activation of autophagy. IFN-γ may activate autophagy through the function of immunity-related GTPases and through the phosphorylation of beclin 1 by death-associated protein kinase 1 (DAPK1).[52] The signaling mechanisms that

autophagy uses for selective degradation of intracellular microorganisms are largely undetermined and more research is needed to elucidate the specific signaling pathways. However, it appears that lipid second messengers and phosphatidylinositol 3-phophate (PI3P) are required for autophagy.[53]

Infection with intracellular *Leishmania amazonensis,* can be a chronic debilitating disease in humans. There are currently no vaccines available and treatment modalities are often harsh. A productive antibody response is important in clearing an infection with *L. amazonensis,* in our in vivo and in vitro models of disease. Modulation of the host immune response for clearance of the intracellular parasites may be a possible host-direct immunotherapy. Activating macrophages to produce the necessary immune factors to kill the intracellular parasite may be a new treatment option that would allow for clearance of the parasite after infection without deleterious side effects. The ability to decrease an infection with *L. amazonensis* requires superoxide production within macrophages, which appears to be linked to the autophagy pathway to parasites for subsequent killing, similar to other intracellular pathogens.

References

1. Felipe Francisco Tuon, V.A.N., Valdir Sabbaga Amato. Leishmania: origin, evolution, and future since the Precambrian. *FEMS immunology and medical microbiology* **54**, 158-166 (2008).

2. Herwaldt, B.L. Leishmaniasis. *Lancet* **354**, 1191-1199 (1999).

3. K Al-Mutawakel, C.S., A Shami, M Sakr, DA Groneberg, and D Quarcoo. Scientometric analysis of the world-wide research efforts concerning Leishmaniasis. *Parasites and Vectors* **3** (2010).

4. WHO. Leishmaniasis. 2014 [cited 2014]Available from:

5. CDC. Leishmaniasis. 2013 [cited 2014]Available from:

6. Ashford, R.W. The leishmaniases as emerging and reemerging zoonoses. *International Journal of Parasitology* **30**, 1269-1281 (2000).

7. Reithinger, R. & Dujardin, J.C. Molecular diagnosis of leishmaniasis: Current status and future applications. *Journal of clinical microbiology* **45**, 21-25 (2007).

8. Monge-Maillo, B. & Lopez-Velez, R. Therapeutic options for visceral leishmaniasis. *Drugs* **73**, 1863-1888 (2013).

9. Monge-Maillo, B. & Lopez-Velez, R. Therapeutic options for old world cutaneous leishmaniasis and new world cutaneous and mucocutaneous leishmaniasis. *Drugs* **73**, 1889-1920 (2013).

10. Afonso, L.C. & Scott, P. Immune responses associated with susceptibility of C57BL/10 mice to Leishmania amazonensis. *Infection and immunity* **61**, 2952-2959 (1993).

11. Sacks, D. & Noben-Trauth, N. The immunology of susceptibility and resistance to Leishmania major in mice. *Nat. Rev. Immunol.* **2**, 845-858 (2002).

12. Gibson-Corley, K.N. *et al.* Promotion of a functional B cell germinal center response after Leishmania species co-infection is associated with lesion resolution. *The American journal of pathology* **180**, 2009-2017 (2012).

13. Lynn Soong, C.A.H., Peter C. Melby. Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis. *Semin Immunopathol* **34**, 735-751 (2012).

14. Mukbel, R., Petersen, C.A. & Jones, D.E. Soluble factors from Leishmania major-specific CD4(+)T cells and B cells limit L-amazonensis amastigote survival within infected macrophages. *Microbes and Infection* **8**, 2547-2555 (2006).

15. Mukbel, R.M. *et al.* Macrophage killing of Leishmania amazonensis amastigotes requires both nitric oxide and superoxide. *American Journal of Tropical Medicine and Hygiene* **76**, 669-675 (2007).

16. Boggiatto, P.M. *et al.* Altered dendritic cell phenotype in response to Leishmania amazonensis amastigote infection is mediated by MAP kinase, ERK. *The American journal of pathology* **174**, 1818-1826 (2009).

17. Soong, L. *et al.* Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to Leishmania amazonensis infection. *Immunity* **4**, 263-273 (1996).

18. Gibson-Corley, K.N., Boggiatto, P.M., Mukbel, R.M., Petersen, C.A. & Jones, D.E. A deficiency in the B cell response of C57BL/6 mice correlates with loss of macrophage-mediated killing of Leishmania amazonensis. *International Journal for Parasitology* **40**, 157-161 (2010).

19. Bhardwaj, S., Srivastava, N., Sudan, R. & Saha, B. Leishmania interferes with host cell signaling to devise a survival strategy. *J Biomed Biotechnol* **2010**, 109189 (2010).

20. Gibson-Corley, K.N., Boggiatto, P.M., Mukbel, R.M., Petersen, C.A. & Jones, D.E. A deficiency in the B cell response of C57BL/6 mice correlates with loss of macrophage-mediated killing of Leishmania amazonensis. *International journal for parasitology* **40**, 157-161 (2010).

21. Mukbel, R., Petersen, C.A. & Jones, D.E. Soluble factors from Leishmania major-specific CD4+T cells and B cells limit L. amazonensis amastigote survival within infected macrophages. *Microbes and infection / Institut Pasteur* **8**, 2547-2555 (2006).

22. Leandro, M.J. B-cell subpopulations in humans and their differential susceptibility to depletion with anti-CD20 monoclonal antibodies. *Arthritis research & therapy* **15 Suppl 1**, S3 (2013).

23. Alduaij, W. & Illidge, T.M. The future of anti-CD20 monoclonal antibodies: are we making progress? *Blood* **117**, 2993-3001 (2011).

24. Hamaguchi, Y. *et al.* The peritoneal cavity provides a protective niche for B1 and conventional B lymphocytes during anti-CD20 immunotherapy in

mice. *Journal of immunology (Baltimore, Md. : 1950)* **174**, 4389-4399 (2005).

25. Montalvao, F. *et al.* The mechanism of anti-CD20-mediated B cell depletion revealed by intravital imaging. *Journal of Clinical Investigation* **123**, 5098-5103 (2013).

26. Aksoy, S., Dizdar, O., Hayran, M. & Harputluoglu, H. Infectious complications of rituximab in patients with lymphoma during maintenance therapy: a systematic review and meta-analysis. *Leukemia & lymphoma* **50**, 357-365 (2009).

27. Kelesidis, T., Daikos, G., Boumpas, D. & Tsiodras, S. Does rituximab increase the incidence of infectious complications? A narrative review. *Int. J. Infect. Dis.* **15**, E2-E16 (2011).

28. Ueno, N. & Wilson, M.E. Receptor-mediated phagocytosis of Leishmania: implications for intracellular survival. *Trends in parasitology* **28**, 335-344 (2012).

29. Galli, S.J., Borregaard, N. & Wynn, T.A. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nature Immunology* **12**, 1035-1044 (2011).

30. Mukbel, R.M. *et al.* Macrophage killing of Leishmania amazonensis amastigotes requires both nitric oxide and superoxide. *The American journal of tropical medicine and hygiene* **76**, 669-675 (2007).

31. Liew, F.Y., Millott, S., Parkinson, C., Palmer, R.M.J. & Moncada, S. MACROPHAGE KILLING OF LEISHMANIA PARASITE INVIVO IS MEDIATED BY NITRIC-OXIDE FROM L-ARGININE. *J. Immunol.* **144**, 4794-4797 (1990).

32. Wei, X.Q. *et al.* ALTERED IMMUNE-RESPONSES IN MICE LACKING INDUCIBLE NITRIC-OXIDE SYNTHASE. *Nature* **375**, 408-411 (1995).

33. McGavin, Z.a. *Pathologic Basis of Veterinary Disease, 5th Edition*, 2012.

34. Bogdan, C., Rollinghoff, M. & Diefenbach, A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Current Opinion in Immunology* **12**, 64-76 (2000).

35. Bhardwaj, S., Srivastava, N., Sudan, R. & Saha, B. Leishmania Interferes with Host Cell Signaling to Devise a Survival Strategy. *Journal of Biomedicine and Biotechnology* (2010).

36. Raghavan, M. Fc Receptors and their interactions with immunoglobulins. *Cell Dev Biol* (1996).

37. Bonetto, S., Spadola, L., Buchanan, A.G., Jermutus, L. & Lund, J. Identification of cyclic peptides able to mimic the functional epitope of IgG1-Fc for human Fc gamma RI. *Faseb Journal* **23**, 575-585 (2009).

38. Harrison, P.T., Davis, W., Norman, J.C., Hockaday, A.R. & Allen, J.M. Binding of monomeric immunoglobulin G triggers Fc gamma RI-mediated endocytosis. *The Journal of biological chemistry* **269**, 24396-24402 (1994).

39. Bonetto, S., Spadola, L., Buchanan, A.G., Jermutus, L. & Lund, J. Identification of cyclic peptides able to mimic the functional epitope of IgG1-Fc for human Fc gammaRI. *FASEB J* **23**, 575-585 (2009).

40. Tse, S.M. *et al.* Differential role of actin, clathrin, and dynamin in Fc gamma receptor-mediated endocytosis and phagocytosis. *J Biol Chem* **278**, 3331-3338 (2003).

41. Pfefferkorn, L.C., Guyre, P.M. & Fanger, M.W. Functional comparison of the inductions of NADPH oxidase activity and Fc gamma RI in IFN gamma-treated U937 cells. *Molecular immunology* **27**, 263-272 (1990).

42. Huang, Z.Y. *et al.* Differential kinase requirements in human and mouse Fc-gamma receptor phagocytosis and endocytosis. *Journal of Leukocyte Biology* **80**, 1553-1562 (2006).

43. Willis, H.E., Browder, B., Feister, A.J., Mohanakumar, T. & Ruddy, S. Monoclonal antibody to human IgG Fc receptors. Cross-linking of receptors induces lysosomal enzyme release and superoxide generation by neutrophils. *Journal of immunology (Baltimore, Md. : 1950)* **140**, 234-239 (1988).

44. Crockett-Torabi, E. & Fantone, J.C. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fc gamma receptor-specific mechanisms. *Journal of immunology (Baltimore, Md. : 1950)* **145**, 3026-3032 (1990).

45. Deretic, V. & Levine, B. Autophagy, immunity, and microbial adaptations. *Cell host & microbe* **5**, 527-549 (2009).

46. Klionsky, D.J. & Emr, S.D. Autophagy as a regulated pathway of cellular degradation. *Science* **290**, 1717-1721 (2000).

47. Simon, D.J.P.a.A.K. Autophagy in the immune system. *Immunology*, 1-8 (2013).

48. Codogno, P., Mehrpour, M. & Proikas-Cezanne, T. Canonical and non-canonical autophagy: variations on a common theme of self-eating? *Nature Reviews Molecular Cell Biology* **13**, 7-12 (2012).

49. Martinez, J. *et al.* Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. *Nat Cell Biol* **17**, 893-906 (2015).

50. Mehta, P., Henault, J., Kolbeck, R. & Sanjuan, M.A. Noncanonical autophagy: one small step for LC3, one giant leap for immunity. *Curr Opin Immunol* **26**, 69-75 (2014).

51. Huang, J. & Brumell, J.H. Bacteria-autophagy interplay: a battle for survival. *Nature Reviews Microbiology* **12**, 101-114 (2014).

52. Gutierrez, M.G. *et al.* Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766 (2004).

53. Shahab Shahnazari, W.-L.Y., Cheryl L. Birmingham, Jessica Shiu, Anton Namolovan, Yiyu T. Zheng, Keiko Nakayama, Daniel J. Klionsky, and John H. Brumell. A Diacyclglycerol-Dependent Signaling Pathway Contributes to Regulation of Antibacterial Autophagy. *Cell Host and Microbe*, 137-146 (2010).

CHAPTER 2

CHARACTERIZATION OF THE B CELL RESPONSE TO *LEISHMANIA* INFECTION AFTER ANTI-CD20 B CELL DEPLETION

A modification of the paper published in *International Journal of Clinical and Experimental Pathology*

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Abstract

Anti-CD20 depletion therapies targeting B cells are commonly used in malignant B cell disease and autoimmune diseases. There are concerns about the ability of B cells to respond to infectious diseases acquired either before or after B cell depletion. There is evidence that the B cell response to existing or acquired viral infections is compromised during treatment, as well as the antibody response to vaccination. Our laboratory has an experimental system using co-infection of C3H mice with both *Leishmania major* and *Leishmania amazonensis* that suggests that the B cell response is important to healing infected mice. We tested if anti-CD20 treatment would completely restrict the B cell response to these intracellular pathogens. Infected mice that received anti-CD20 B cell depletion therapy had a significant decrease in $CD19^{+}$ cells within their lymph nodes and spleens. However, splenic B cells were detected in depleted mice and an antigen-specific antibody response was produced. These results indicate that an antigen-specific B cell response towards intracellular pathogens can be generated during anti-CD20 depletion therapy.

Keywords: B cell, CD20, *Leishmania*, immunohistochemistry

Introduction

CD20 is a B-cell specific antigen that is expressed solely on B cells but not on early progenitor B cells. CD20 is present on the B cell from the pre-B cell stage until the mature B cell stage and is excluded on plasmablasts and plasma cells. CD20 is thought to be involved in B cell activation and differentiation.[1, 2] Antibodies against CD20 were developed in the late 1980s and 1990s for the treatment of follicular lymphoma, non-Hodgkin's lymphoma, and cancers of the B-cell lineage.[3] Rituximab, an anti-CD20 monoclonal antibody (Rituxan©, Genentech, Biogen IDEC), was first licensed for use in 1997 against follicular lymphoma and is now licensed for use against Non-Hodgkin's Lymphoma (NHL), Chronic Lymphocytic Leukemia (CLL), Rheumatoid Arthritis (RA), Granulomatosis with Polyangitis (GPA or Wegener's Granulomatosis), and Microscopic Polyangitis (MPA). Other anti-CD20 mAbs have also been developed, including Ofatumumab, Ocrelizumab, Veltuzumab, AME-133v, PRO131921, and GA101.[3] Anti-CD20 monoclonal antibodies are most commonly used in therapies against lymphoma and autoimmune disorders, often in combination with other immunosuppressive agents. Infections are among the most important causes of morbidity and mortality in patients suffering from cancer; however, there is a lack of research on the immunosuppression that anti-CD20 therapies may provoke and whether these therapies may exacerbate these secondary infections.[4]

Although anti-CD20 therapies have a proven efficacy and safety, the question remains as to whether these therapies lead to an increased susceptibility to numerous infections. Many clinical trials have conflicting results regarding the association of anti-CD20 therapy and infections; however, one study has shown an increased incidence of infections in patients with lymphomas and rheumatoid arthritis receiving rituximab.[5] A review of recent data showed that rituximab therapy significantly increases the risk of infection in patients with lymphoma or other hematological malignancies; however, this increased risk of infection was comparable to other treatments used in patients with rheumatoid arthritis.[6] Often, patients receiving anti-CD20 therapies have other complicating factors including other active infections.[6] Studies have shown that although anti-CD20 therapies induce B-cell depletion for 6 to 9 months, their immunoglobulin levels do not decrease.[7] This phenomenon may be due to the presence of long-lived plasma cells. Plasma cells can survive for periods greater than 1 year, even in the absence of a memory B cell population.[8] Therefore, most patients receiving B-cell depletion therapy do not have an increase in the number of infectious complications.[7] Other studies have shown that patients receiving anti-CD20 therapy have an impaired humoral immune response to a primary antigen but not to a recall antigen.[7, 9, 10] Although these studies show an impaired humoral immune response, many patients receiving anti-CD20 therapy still produced a measurable antibody response to vaccination and approximately 20% of the patients were seroprotected.[9] Nothing is known about the antibody response towards intracellular pathogens during anti-CD20 treatment. Although it

is often thought that humoral immunity is, at best, inconsequential towards intracellular pathogens or, at worst, detrimental to immune control of intracellular pathogens there is emerging evidence that antibodies can play a role in the protective response to infection with intracellular pathogens.[11, 12, 13, 14]

Leishmaniasis is a commonly used immunologic model of chronic infectious disease. *Leishmania* species are obligate intracellular protozoan parasites transmitted by the bite of a sand fly. *L. major* and *L. amazonensis* cause cutaneous leishmaniasis in many mammalian species, and *L. amazonensis* can lead to non-healing lesions.[15] Leishmaniasis is prevalent in 98 countries in the tropics and subtropics and is considered a neglected tropical disease. Multiple mouse models of leishmaniasis are commonly used to study host-pathogen dynamics. C3HeB/FeJ (C3H) mice infected with *L. major* will resolve cutaneous lesions within 8 to 12 weeks whereas the same mouse strain infected with *L. amazonensis* develops non-healing cutaneous lesions. However, mice co-infected with *L. major* and *L. amazonensis* resolve their lesions. Our lab has discovered that $CD4^+$ T cells and $CD19^+$ B cells from *L. major*-infected C3H mice are necessary to kill *L. amazonensis* within infected macrophages in an *in vitro* assay.[12, 16, 17] We wanted to test the ability of anti-CD20 administration to prevent a detectable *Leishmania*-specific B cell response to determine if we could use this model in our experimental system. Treatment with monoclonal anti-CD20 antibodies have been associated with approximately 99% depletion of normal B cells in peripheral blood[18]. However, we show that C3H mice co-infected with *L. major* and *L. amazonensis* and treated with anti-CD20 mAb still

have a B cell response to these intracellular parasites. Although the depleted mice had significantly less CD19+ cells in the lymph nodes and spleen they still had some germinal center formation and detectable antibodies via immunoblotting. In this report we determine the ability of the mouse to mount an effective immune response to an intracellular infection during monoclonal anti-CD20 treatments.

Materials and Methods

Mice

C3HeB/FeJ (C3H) mice (8-10 weeks of age) were obtained from an in-house breeding colony and maintained in a specific pathogen-free facility. Mice were infected with either 5 x 10^6 stationary phase *L. major,* 5 x 10^6 stationary phase *L. amazonensis* or 2.5 x 10^6 *L. major* (LM) and 2.5 x 10^6 *L. amazonensis* (LA) promastigotes in 50 µL of PBS in the left hind footpad. In the first experiment there were a total of 25 mice with 5 mice per treatment group: 1) LA infected mice 2) LM infected mice 3) LM infected and anti-CD20 treated 4) co-infected, and 5) co-infected and anti-CD20 treated. In the second experiment there were 20 mice total with 5 mice per treatment group: 1) uninfected 2) uninfected and anti-CD20 treated 3) co-infected and 4) co-infected and anti-CD20 treated. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Iowa State University. Lesion size was monitored and the results were expressed as the difference between the footpad thickness for the uninfected foot and the footpad thickness for the infected foot.

B-cell depletion

Mice were given intravenous injections of 200 µg anti-CD20 mAb (IgG1) provided by Biogen IDEC (Cambridge, MA) or 200ug of IgG1 isotype control (BioXcell, West Lebanon, NH) two weeks post-infection. Mice were then given intraperitoneal injections of anti-CD20 mAb or IgG1 isotype control every 2 weeks for a total of 3 treatments.[19]

Parasites and Antigens

L. amazonensis (MHOM/BR/00/LTB0016) and *L. major* (MHOM/IL/80/Friedlin) promastigotes were grown in complete Grace's medium (Atlanta Biologicals, Lawrencville, GA) to stationary phase, harvested, washed in endotoxin free PBS (Cellgro, Herdon, VA) and prepared to a concentration of 1 x 10^8 parasites/ml. Freeze-thawed *Leishmania* antigen was obtained from stationary phase promastigotes as previously described.[20]

Flow Cytometry

For flow cytometry analysis of surface molecule expression, 1 x 10^6 total draining lymph node cells or splenocytes were washed in 2 ml of fluorescence-activated cell sorting buffer (FACS, 0.1% sodium azide and 0.1% bovine serum albumin in phosphate buffer saline). Fcγ receptors were blocked with 10% purified rat anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA) in 1 mg/ml rat IgG for 20 minutes at 4°C to prevent non-specific binding. Cells were then

incubated with the appropriate antibody or isotype control for 30 minutes on ice in the dark. The antibodies used include phycoerythrin-labeled CD19 and phycoerythrin-labeled rat IgG2a isotype control. Antibodies were purchased from BD Pharmingen (San Diego, CA). Following staining, cells were washed in 2 ml of FACS buffer and fixed in 200 µl of 1% paraformaldehyde and stored at 4°C until analysis. Analysis was performed on a BD FACScanto flow cytometer (Becton Dickinson, San Jose, CA), and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Lymph node and Spleen Histopathology and Immunohistochemistry

Spleens and popliteal lymph nodes from the left hind leg draining the site of infection were harvested and placed in cassettes in 10% neutral buffered formalin for histological and immunohistochemical analyses. Histological examination was performed on paraffin-embedded tissues cut at 5-µm thickness onto positively charged slides and stained with H&E. For immunohistochemistry, slides were de-paraffinized and blocked with 20% normal rabbit serum. The sections were then incubated with either a rat anti-mouse B220/CD45R antibody (BD Harlingen, San Diego, CA) at a concentration of 1:50 or biotin-labeled PNA (Vector Laboratories, Burlingame, CA) at a concentration of 1:100 in 10% normal rabbit serum. The slides were rinsed with PBS and then incubated with biotin-labeled goat anti-rat IgG (KPL, Gaithersburg, MD) at a concentration of 1:500 in TBS for the B220/CD45R labeled slides. Slides were washed and incubated with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) for 15 minutes.

After 2 PBS washes, the color was developed with Nova Red (KPL, Gaithersburg, MD). The slides were then counterstained with Harris' hematoxylin, then dehydrated and mounted with coverslips.

Immunoblot Analysis

Protein content of all cell extracts was determined via BCA protein assay (Pierce, Rockford, IL) according to manufacturer's recommendations, and all samples were normalized to 2 mg/ml using distilled water. Samples (20 to 30 µg of protein) were heated for 4 minutes at 95°C in 4x loading buffer and electrophoresis was performed on a 12% SDS-polyacrylamide electrophoresis gel. Gels were transferred onto polyvinylidene fluoride membranes, blocked with 5% dry milk, and probed with pooled serum from mice. Signals were detected with horseradish-peroxidase-conjugated goat anti-rabbit antibodies (1:20,000) (Jackson ImmunoResearch, West Grove, PA) using the SuperSignal West chemiluminescent substrate (Pierce, Rockford, IL) and signal was detected with radiography film (Midsci, St. Louis, MO).

Statistics

Statistical analysis was performed with Prism5 (Graph-Pad Software Inc., La Jolla, CA). Differences between groups were determined using unpaired t-tests or a Mann-Whitney *U*-test when appropriate. *P* values <0.05 were considered statistically significant.

Results

C3H mice co-infected with *L. major* and *L. amazonensis* resolve cutaneous lesions following B cell depletion

We have previously demonstrated that C3H mice will heal a co-infection with *L. major* and *L. amazonensis* by 10 to 12 weeks post-infection.[11] However, we have also shown that a productive B cell response correlates with healing a co-infection with *L. major* and *L. amazonensis*.[12] We tested the hypothesis that anti-CD20 treatment would eliminate a B cell response to infection with these intracellular parasites. We infected mice with *L. major* and *L. amazonensis* and at 2 weeks post-infection, began administration of B cell depletion therapies, as described above. We observed no significant differences in the kinetics of lesion development between co-infected mice that received the anti-CD20 or isotype control treatment throughout the time course of the study (Figure 1). In addition, lesion development in the co-infected groups was similar to the kinetics of lesion development in *L. major*-infected mice treated with anti-CD20 or isotype control antibodies (data not shown). Altogether, these data indicate that B cell depletion had no effect on lesion development of either co-infected or *L. major*-infected mice.

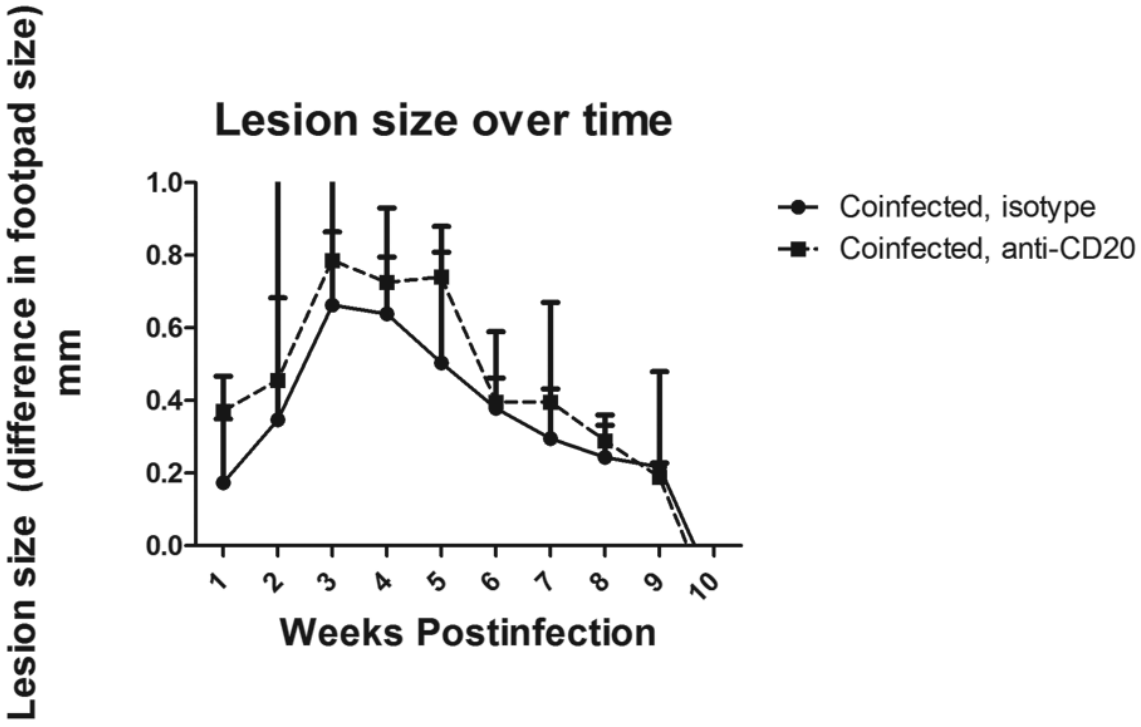

Figure 1. Co-infection of C3HeB/FeJ (C3H) mice with *Leishmania major* (LM) and *L. amazonensis* (LA) allows for lesion resolution by 10 weeks post-infection irrespective of B cell depletion therapy. Mice were inoculated with LA and LM stationary phase promastigotes in the left hind footpad. Mice were then treated with anti-CD20 or an isotype control at two weeks post infection and every two weeks until the end of study. Data are representative of two separate experiments.

CD20 depleted C3HeB/FeJ mice have significantly fewer CD19$^+$ cells present in draining lymph nodes and spleens

In order to determine how the anti-CD20 antibody treatment affected B cells, we analyzed draining lymph node and spleen cell homogenates at 10 weeks post-infection by flow cytometry with anti-CD19 to identify B cells. We used anti-CD19 instead of anti-CD20 or anti-B220 to avoid antibody interference and to allow us to differentiate B cells from plasmacytoid dendritic cells, which also express

B220.[21] CD19, in conjunction with CD21 and CD81, is part of the B cell co-receptor.[22] CD19, like CD20 is present on B cells throughout the developmental stages, excluding plasmablasts and plasma cells. [23] At 10 weeks the percentage of $CD19^+$ B cells from the draining lymph nodes or spleen of anti-CD20-treated mice was significantly less as compared to isotype-treated mice (Figure 2A and 2B). Overall, the percentage of $CD19^+$ B cells in the spleen and draining lymph node of isotype-treated mice were similar, 40% and 50% respectively. However, post-treatment, we observed a 97.5% reduction of CD19+ B cells in the draining lymph node of anti-CD20 treated mice, but only an 80% reduction in the percentage of CD19+ B cells in the spleen of treated mice (Figure 2A and B). These data would suggest that anti-CD20 antibody treatment differentially affects B cell depletion, with the spleen being less affected by the treatment. Similar results were found in *L. major* infected mice with more depletion in the lymph nodes as compared to the spleen (data not shown).

To further assess the B cell response, we also compared total lymphocyte counts from lymph nodes and spleens of co-infected mice with and without anti-CD20 treatment. Co-infected mice treated with anti-CD20 had significantly less lymph node cells and splenocytes compared to the isotype controls (Figure 2C). This was also true for *L. major*-infected mice treated with anti-CD20 (data not shown).

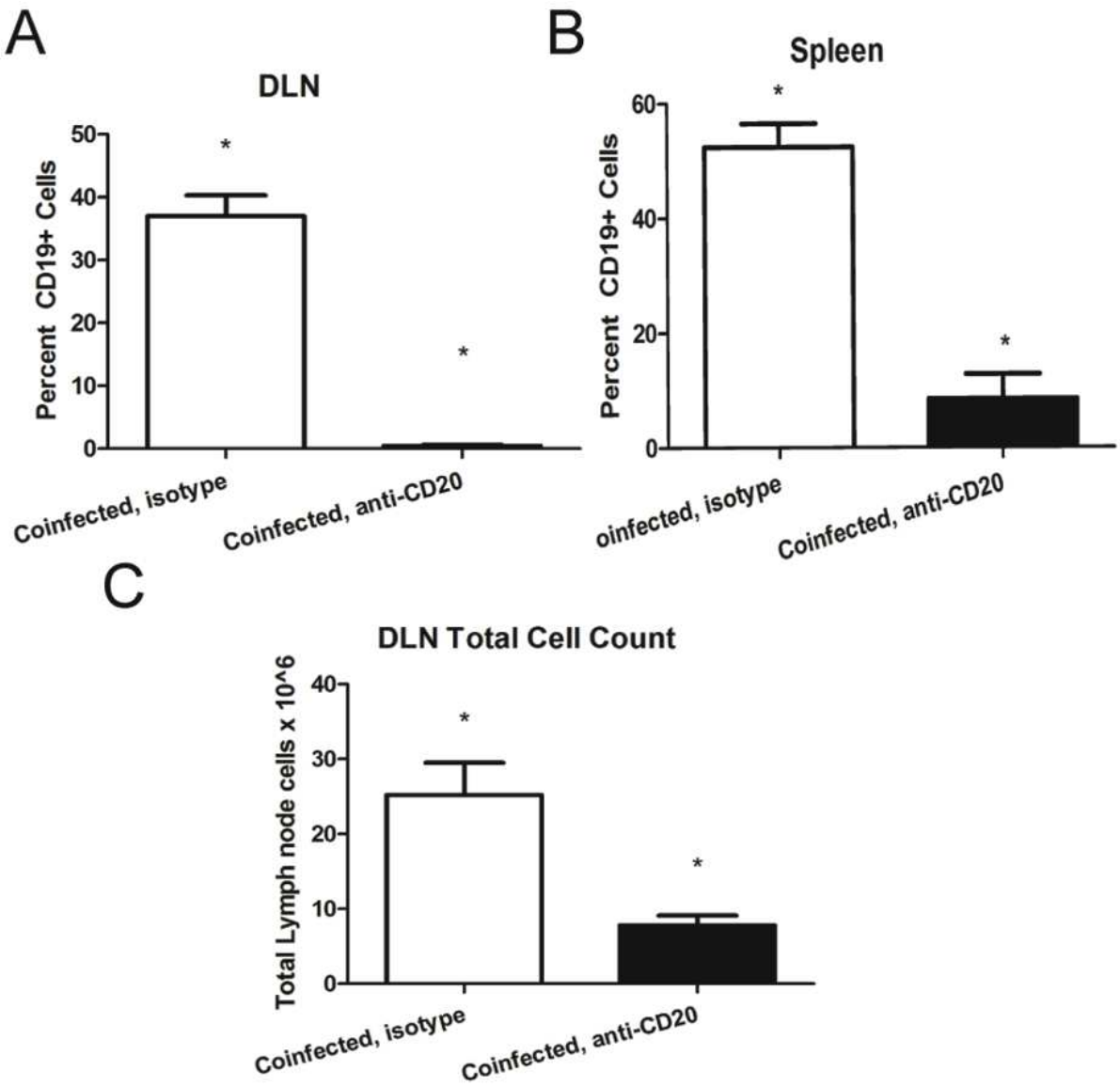

Figure 2. Mice co-infected with *Leishmania major* (LM) and *L. amazonensis* (LA) that received anti-CD20 monoclonal antibody therapy had significantly less $CD19^+$ cells and significantly fewer total lymphocytes within the draining lymph nodes as compared to the isotype control. Total draining lymph node cells and splenocytes were harvested at 10 weeks post-infection. Cells were analyzed via surface expression of CD19. (A, B, C). Data are the mean +/- SEM of two separate experiments. A, B *P<0.0001, C *P=0.0048 (t-test).

C3HeB/FeJ mice depleted with anti-CD20 have smaller lymph nodes and altered lymph node histomorphology

Next we characterized the histomorphology of draining lymph nodes and spleens from anti-CD20 and isotype control-treated mice 10 weeks post-infection with *L. major* and *L. amazonensis*. Lymph nodes from co-infected mice had reactive lymphoid hyperplasia with a sinus histiocytosis. There was diffuse expansion of the cortex due to marked proliferation of lymphocytes within the paracortex, follicles, and medullary cords. There was also hypercellularity of the medullary sinuses composed of infiltrates of inflammatory cells consisting mainly of macrophages with fewer lymphocytes, plasma cells, and neutrophils (Figure 3).

Draining lymph nodes from anti-CD20 treated mice were significantly smaller as compared to the isotype-treated controls (Figure 3). Anti-CD20 treatment also resulted in a loss of normal lymph node architecture and a lack of follicular organization. There was accentuation of the lymph node stroma, including numerous fibroblasts and adipose cells admixed with lymphocytes, plasma cells, and fewer neutrophils (Figure 3).

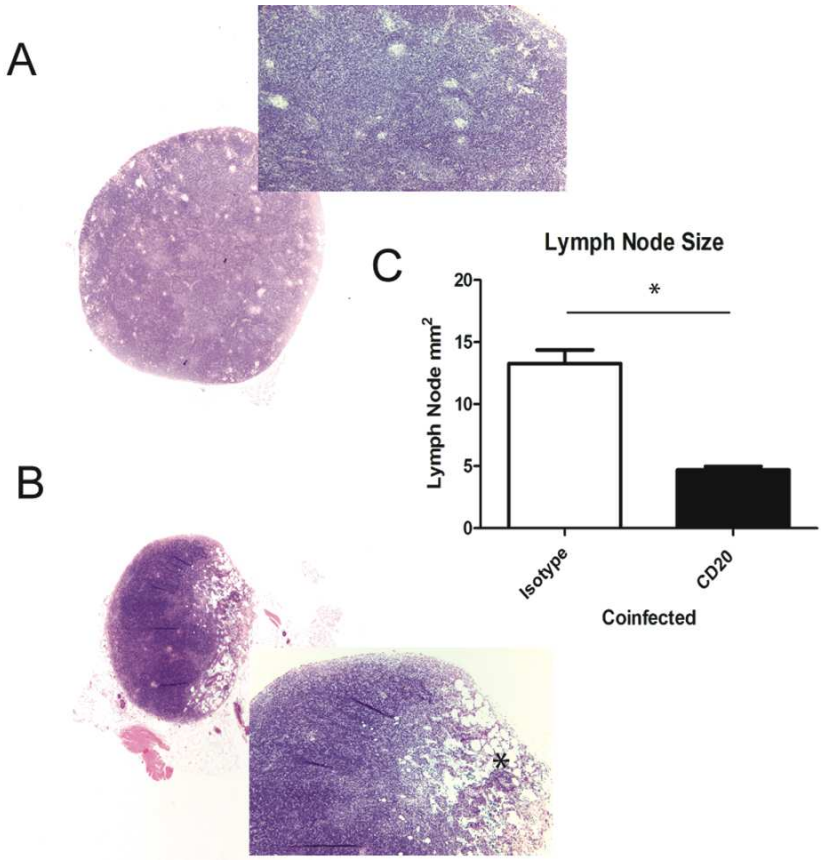

Figure 3. Mice co-infected with *Leishmania major* (LM) and *L. amazonensis* (LA) that received anti-CD20 monoclonal antibody therapy had immunopathology and significantly smaller draining lymph nodes. Draining lymph nodes and spleens were harvested at 10 weeks post-infection and placed in 10% neutral buffered formalin. Histological examination was performed on paraffin-embedded tissues cut at 5-µm thickness onto positively charged slides and stained with H&E. Co-infected mice that did not receive anti-CD20 had a reactive lymphoid hyperplasia with a sinus histiocytosis (A). Co-infected mice that received anti-CD20 treatment had small lymph nodes with accentuation of the stromal fibroblasts and adipose cells (*) (B). The lymph node size was measured using CellSens Standard 1.9 (Olympus) (C). Data are the mean +/- SEM of two separate experiments. *P=0.0003 (t-test).

C3HeB/FeJ mice depleted with anti-CD20 have significantly less B220/CD45R positive immunostaining and a significant difference in the number of germinal centers in the draining lymph node

Anti-B220/CD45R and biotin peanut agglutinin (PNA) immunohistochemistries were performed to compare B cell populations and germinal center formation, respectively between the B cell depleted and isotype-treated groups of co-infected mice. Mice that were B cell depleted had significantly less B220/CD45R immunostaining as compared to the isotype-treated groups (Figure 4). B220/CD45R is predominantly expressed on all B lymphocytes, including pro, mature, and activated B cells, and most $B220^{+}$ cells are also $CD19^{+}$.

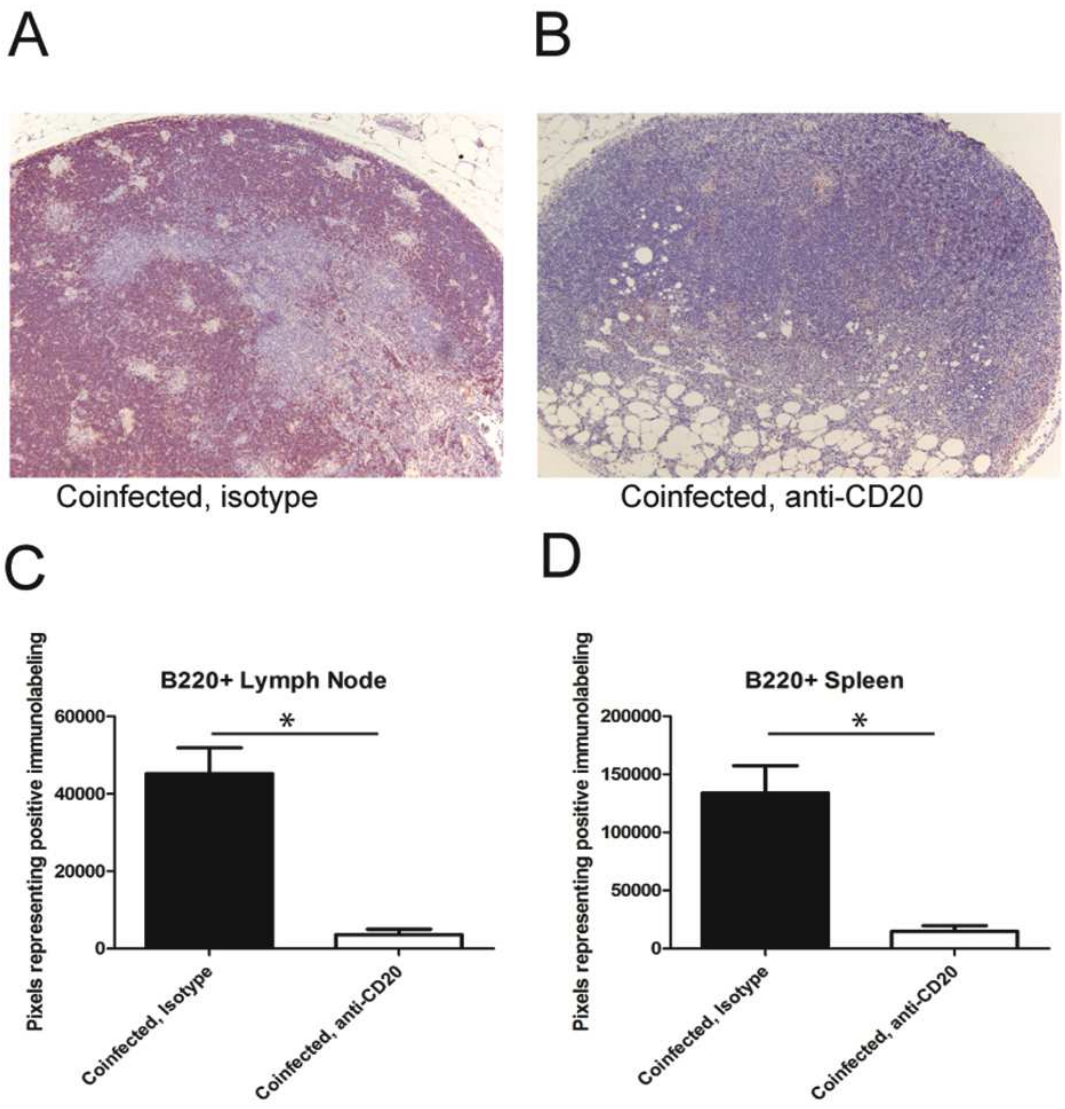

Figure 4. Mice co-infected with *Leishmania major* (LM) and *L. amazonensis* (LA)

that received anti-CD20 therapy had significantly less immunostaining for B220/CD45R. Co-infected mice that did not receive anti-CD20 treatment had significantly more $B220^{+}$ immunostaining in both the draining lymph node (A, C) and spleen (D). Co-infected mice that received anti-CD20 treatment had very little positive immunostaining for B220/CD45R (B). Data are representative of two separate experiments +/- SEM. *P<0.0001 (t-test).

Biotin peanut agglutinin (PNA) is generally used to identify mature lymphocytes in germinal centers. The germinal center score was significantly different between the isotype control and anti-CD20 treated draining lymph nodes. There were few small PNA positive germinal centers throughout the isotype control (Figure 5A), but no germinal centers were seen in the anti-CD20 treated draining lymph nodes (Figure 5B). However, there were no significant differences in the germinal center scores of the spleens between co-infected mice that had been treated with anti-CD20 or an isotype-control antibody. There were very low numbers of germinal centers in the isotype control spleens at 10 weeks post-infection, despite finding only one germinal center in an anti-CD20 treated mouse spleen.

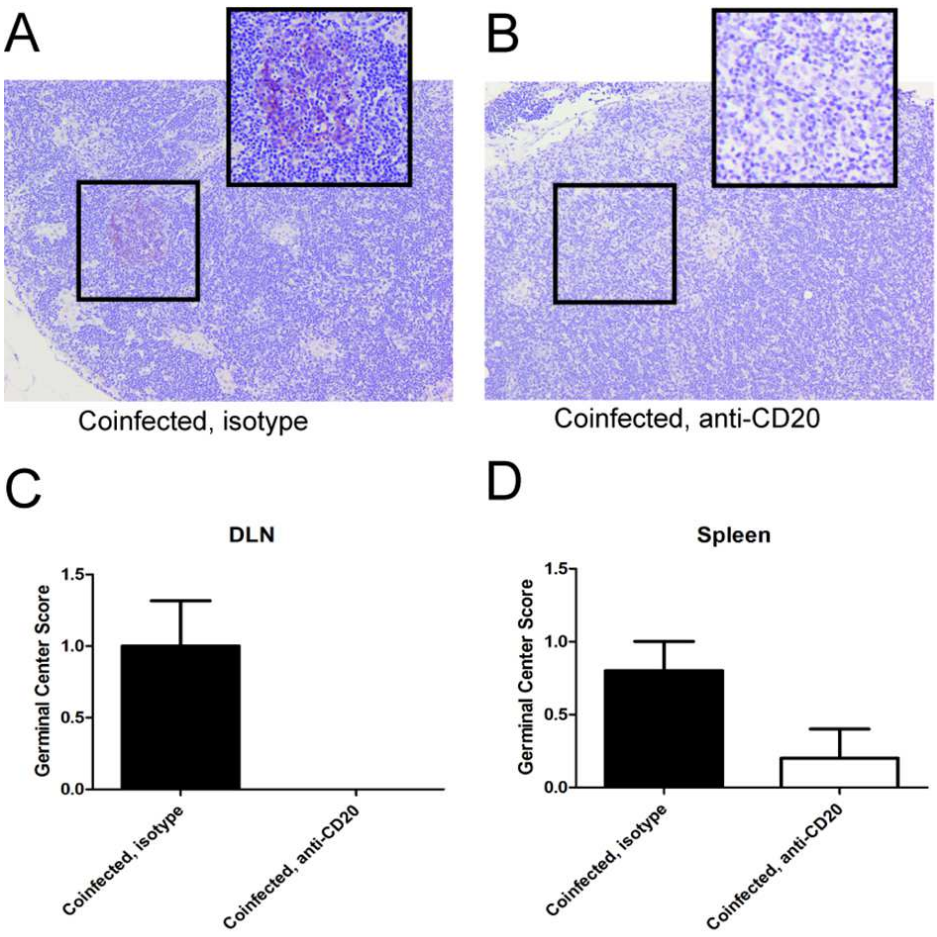

Figure 5. Mice co-infected with *Leishmania major* (LM) and *L. amazonensis* (LA) that received anti-CD20 therapy had a significant decrease in the number of germinal centers in the draining lymph nodes but not the spleen. Photomicrographs of biotin peanut agglutinin (PNA) staining highlights a germinal center (A, inset) within a control mouse compared to negative immunostaining within a B-cell depleted lymph node (B, inset). Histological germinal center scores for PNA immunoreactivity were performed at 10 weeks post-infection. Score is based on the number of PNA^+ germinal centers within the tissue section of a single draining lymph node. Data are representative of two separate experiments +/- SEM. *P=0.0400 (C), P=0.930 (D) (Mann-Whitney *U* test).

C3HeB/FeJ mice treated with anti-CD20 mAb produce a specific antibody response

Based on our data thus far, anti-CD20 treatment resulted in depletion of B cells from both the draining lymph node and spleen, with the spleen containing readily detectable populations of B cells and a rare germinal center. We hypothesized that perhaps, despite the observed B cell depletion, there was still a detectable antigen-specific antibody response. In order to test this, we performed an immunoblot with serum collected from the co-infected mice treated with anti-CD20 or isotype control antibody, at 10 weeks post-infection. The immunoblot was performed for total IgG, IgG2a, and IgG1. The depleted mice still produced *L. major* and *L. amazonensis* specific antibodies (Figure 6).

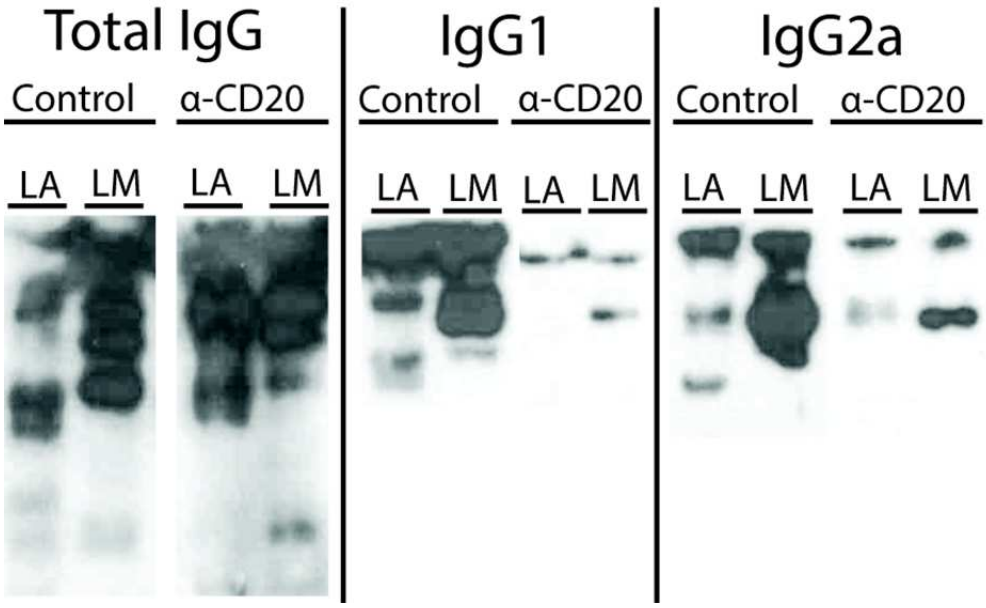

Figure 6. Co-infected mice treated with anti-CD20 still produce parasite-specific antibodies. Western blot analysis of parasite-specific production of total IgG and isotypes IgG1 and IgG2a were performed at 10 weeks post-infection. Freeze-thawed *Leishmania major* (LM) and *L. amazonensis* (LA) antigen were separated on a polyacrylamide gel and protein was transferred to a polyvinylidene fluoride

(PVDF) membrane. The blots were subsequently hybridized with mouse serum (1:25 dilution) pooled from 5 C3H control mice and 5 C3H B-cell depleted mice that were co-infected with *L. major* (LM) and *L. amazonensis* (LA). Following serum hybridization the membranes were probed with goat anti-mouse antibodies to total IgG, IgG1, or IgG2a. Results are from one experiment at 10 weeks post-infection.

Discussion

The work presented in this manuscript demonstrates that mice co-infected with *L. major* and *L. amazonensis* followed by treatment with monoclonal anti-CD20 antibodies resolved cutaneous lesions with normal kinetics (Figure 1). Anti-CD20 antibody treatment resulted in a 97.5% reduction of $CD19^+$ B cells in draining lymph node and an 80% reduction in the spleen compared to isotype controls (Figure 2A & B). Anti-CD20 treatment led to significantly less total lymphocytes (Figure 2C), significantly smaller lymph nodes (Figure 3), significantly less $B220^+$ immunostaining (Figure 4), and significantly fewer germinal centers within draining lymph nodes (Figure 5). However, treated mice still had detectable antigen-specific antibodies to *L. major* and *L. amazonensis* antigens. Given these results, we have demonstrated that mice treated with anti-CD20 depletion antibodies at 2 weeks post-infection retain a population of responsive B cells and are capable of developing a detectable antibody response to the intracellular pathogen.

In this study mice were treated with anti-CD20 antibody therapy at 2 weeks post-infection to ensure a proper $CD4^+$ T cell response. Other studies have shown that B cells regulate the initial proliferation of $CD4^+$ T cells after encounter with antigen, influence the maintenance of CD4+ T cells, have roles in

$CD4^+$ T cell memory responses, and can influence regulatory T cell numbers and function.[24, 25, 26] Therefore, we did not begin B cell depletion therapy before infection, to ensure the functionality of the effector $CD4^+$ T cell populations that are needed to resolve cutaneous leishmaniasis.

It is possible that the mice were able to mount an effective humoral immune response to the *Leishmania* antigens within the first 2 weeks before depletion therapy began. This may have lead to the development of long-lived antigen-specific plasma cells that would not be affected by the anti-CD20 therapy. The anti-CD20 dose we used was comparable to other murine studies that demonstrated B cell depletion with some studies depleting essentially all splenic B cells.[27, 28] Our experimental system resulted in only an 80% reduction of B cells in the spleen. In addition, our data differs from some articles that have shown that bone marrow and spleen are more easily depleted than lymph nodes.[1, 28, 29] We expected a more robust depletion and although we did not have an opportunity to test it, a reasonable hypothesis is that the decreased efficiency in depletion may be because of the ongoing chronic intracellular infection. It would be interesting to determine if splenic B cells are relatively resistant to anti-CD20 depletion during a chronic infection, in contrast to draining lymph node B cells which had a 97.5% reduction in B cells. Many factors can influence depletion, including the drug dose administered, distribution to tissues, B-cell intrinsic and microenvironment factors affecting recruitment of effector mechanisms and antigen and effector modulation.[1, 29]

Other possible explanations for the lack of total depletion of functional B cells include the presence of $CD20^+$ B cells that are resistant to anti-CD20 therapies, such as marginal zone B lymphocytes, which have been suggested to be resistant to rituximab depletion.[30] The site at which the B cells are located may also play a large role in the ability of the anti-CD20 depletion therapies. These differences by location may be due to the body's ability to clear antibody targeted cells or the lack of FcR^+ cells that are able to clear the targeted cells.[18]

Another less likely theory that could account for the development of antibodies includes the presence of a $CD20^-$ or $CD20^{low}$ population of B cells that are not depleted by conventional B cell depletion therapies with anti-CD20 antibodies that are still able to produce an antibody response. This may explain the different pattern of immunoglobulins seen on the immunoblots (Figure 6). Regardless, our results support other studies that have shown that despite B cell depletion therapy patients are still able to mount a humoral immune response to a primary antigen, although that response may be impaired.[7, 9, 10, 31] In our experimental infection we show that mice were able to produce a demonstrable B cell response to an intracellular pathogen despite treatment with anti-CD20 antibodies. These results precluded our ability to definitively test the role of B cells during *Leishmania* co-infection.

To our knowledge, the work presented here describes, for the first time, the parameters of the B cell response to *Leishmania* infection after anti-CD20 B cell depletion. We showed that despite B cell depletion, the infected mouse is still able to mount a pathogen-specific humoral immune response. Since B cell

depletion therapy is common in people and is often used in conjunction with other immunosuppressive therapies, further research is necessary to determine the risks of B cell depletion therapy in patients with simultaneous infections and the possible differential susceptibility of B cell subpopulations to depletion during chronic infection. Therefore, further understanding of this model could better reflect some real life scenarios, in which those needing B cell therapy also have concurrent infections or established infections prior to treatment.

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References

1. Leandro, M.J. B-cell subpopulations in humans and their differential susceptibility to depletion with anti-CD20 monoclonal antibodies. *Arthritis research & therapy* **15 Suppl 1**, S3 (2013).

2. Roberts, W.K. *et al.* Vaccination with CD20 peptides induces a biologically active, specific immune response in mice. *Blood* **99**, 3748-3755 (2002).

3. Alduaij, W. & Illidge, T.M. The future of anti-CD20 monoclonal antibodies: are we making progress? *Blood* **117**, 2993-3001 (2011).

4. Lanini, S. *et al.* Risk of infection in patients with lymphoma receiving rituximab: systematic review and meta-analysis. *BMC medicine* **9**, 36 (2011).

5. Aksoy, S., Dizdar, O., Hayran, M. & Harputluoglu, H. Infectious complications of rituximab in patients with lymphoma during maintenance therapy: a systematic review and meta-analysis. *Leukemia & lymphoma* **50**, 357-365 (2009).

6. Kelesidis, T., Daikos, G., Boumpas, D. & Tsiodras, S. Does rituximab increase the incidence of infectious complications? A narrative review. *Int. J. Infect. Dis.* **15**, E2-E16 (2011).

7. van der Kolk, L.E., Baars, J.W., Prins, M.H. & van Oers, M.H.J. Rituximab treatment results in impaired secondary humoral immune responsiveness. *Blood* **100**, 2257-2259 (2002).

8. Slifka, M.K., Antia, R., Whitmire, J.K. & Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363-372 (1998).

9. Kim, W. *et al.* Reduced antibody formation after influenza vaccination in patients with neuromyelitis optica spectrum disorder treated with rituximab. *European journal of neurology : the official journal of the European Federation of Neurological Societies* **20**, 975-980 (2013).

10. Pescovitz, M.D. *et al.* Effect of rituximab on human in vivo antibody immune responses. *J. Allergy Clin. Immunol.* **128**, 1295-U1655 (2011).

11. Gibson-Corley, K.N., Boggiatto, P.M., Mukbel, R.M., Petersen, C.A. & Jones, D.E. A deficiency in the B cell response of C57BL/6 mice correlates with loss of macrophage-mediated killing of Leishmania amazonensis. *International journal for parasitology* **40**, 157-161 (2010).

12. Gibson-Corley, K.N. *et al.* Promotion of a functional B cell germinal center response after Leishmania species co-infection is associated with lesion resolution. *The American journal of pathology* **180**, 2009-2017 (2012).

13. Gibson-Corley, K.N. *et al.* An In Vitro Model of Antibody-Enhanced Killing of the Intracellular Parasite Leishmania amazonensis. *PLoS One* **9**, e106426 (2014).

14. Chan, J. *et al.* The role of B cells and humoral immunity in Mycobacterium tuberculosis infection. *Seminars in immunology* **26**, 588-600 (2014).

15. Tolouei, S., Hejazi, S.H., Ghaedi, K., Khamesipour, A. & Hasheminia, S.J. TLR2 and TLR4 in cutaneous leishmaniasis caused by L. major. *Scandinavian journal of immunology* (2013).

16. Mukbel, R., Petersen, C.A. & Jones, D.E. Soluble factors from Leishmania major-specific CD4+T cells and B cells limit L. amazonensis amastigote survival within infected macrophages. *Microbes and infection / Institut Pasteur* **8**, 2547-2555 (2006).

17. Vanloubbeeck, Y. & Jones, D.E. Protection of C3HeB/FeJ mice against Leishmania amazonensis challenge after previous Leishmania major infection. *The American journal of tropical medicine and hygiene* **71**, 407-411 (2004).

18. Hamaguchi, Y. *et al.* The peritoneal cavity provides a protective niche for B1 and conventional B lymphocytes during anti-CD20 immunotherapy in mice. *Journal of immunology (Baltimore, Md. : 1950)* **174**, 4389-4399 (2005).

19. Yu, S.G., Dunn, R., Kehry, M.R. & Braley-Mullen, H. B cell depletion inhibits spontaneous autoimmune thyroiditis in NODA-2h4 mice. *J. Immunol.* **180**, 7706-7713 (2008).

20. Jones, D.E., Ackermann, M.R., Wille, U., Hunter, C.A. & Scott, P. Early enhanced Th1 response after Leishmania amazonensis infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infection and immunity* **70**, 2151-2158 (2002).

21. Medina, K.L. *et al.* Separation of plasmacytoid dendritic cells from B-cell-biased lymphoid progenitor (BLP) and Pre-pro B cells using PDCA-1. *PloS one* **8**, e78408 (2013).

22. Del Nagro, C.J. *et al.* CD19 function in central and peripheral B-cell development. *Immunologic research* **31**, 119-131 (2005).

23. Katz, B.Z. & Herishanu, Y. Therapeutic targeting of CD19 in hematological malignancies: past, present, future and beyond. *Leukemia & lymphoma* **55**, 999-1006 (2014).

24. Lund, F.E. & Randall, T.D. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nature reviews. Immunology* **10**, 236-247 (2010).

25. Misumi, I. & Whitmire, J.K. B cell depletion curtails CD4+ T cell memory and reduces protection against disseminating virus infection. *Journal of immunology (Baltimore, Md. : 1950)* **192**, 1597-1608 (2014).

26. Bouaziz, J.D. *et al.* Therapeutic B cell depletion impairs adaptive and autoreactive CD4+ T cell activation in mice. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 20878-20883 (2007).

27. Hamel, K.M. *et al.* B cell depletion enhances T regulatory cell activity essential in the suppression of arthritis. *Journal of immunology (Baltimore, Md. : 1950)* **187**, 4900-4906 (2011).

28. Moritoki, Y. *et al.* B-cell depletion with anti-CD20 ameliorates autoimmune cholangitis but exacerbates colitis in transforming growth factor-beta receptor II dominant negative mice. *Hepatology* **50**, 1893-1903 (2009).

29. Vugmeyster, Y., Howell, K., McKeever, K., Combs, D. & Canova-Davis, E. Differential in vivo effects of rituximab on two B-cell subsets in cynomolgus monkeys. *International immunopharmacology* **3**, 1477-1481 (2003).

30. Gong, Q. *et al.* Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. *Journal of immunology (Baltimore, Md. : 1950)* **174**, 817-826 (2005).

31. Bedognetti, D. *et al.* Impaired Response to Influenza Vaccine Associated with Persistent Memory B Cell Depletion in Non-Hodgkin's Lymphoma Patients Treated with Rituximab-Containing Regimens. *J. Immunol.* **186**, 6044-6055 (2011).

CHAPTER 3

SOLUBLE IMMUNE COMPLEXES PROMOTE ANTIBODY-ENHANCED INTRACELLULAR KILLING OF *LEISHMANIA AMAZONENSIS* VIA UPREGULATION OF AUTOPHAGY

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Abstract

The intracellular protozoal parasite *Leishmania* can cause cutaneous and visceral leishmaniasis, a vector borne disease that infects approximately 1 million people each year. C3H mice infected with *L. amazonensis* develop chronic cutaneous lesions with large parasite loads. Using an in vitro assay with immune cells from infected mice we have previously shown that macrophage activation in response to soluble IgG2a immune complexes, IFN-γ and parasite antigen was effective in killing intracellular *L. amazonensis*. Parasite killing was dependent upon FcRγ common-chain and NADPH oxidase-generated superoxide from infected macrophages. Here we show that antibody-enhanced intracellular killing is associated with an upregulation of autophagy as determined by an increase in LC3II and colocalization of LC3 with parasites within parasitophorous vacuoles. LC3 colocalization and superoxide production were dependent upon the PKCδ pathway. These experiments define a new mechanism by which antibodies can promote killing of an intracellular pathogen post-infection.

Introduction

Leishmaniasis is a vector-borne intracellular protozoan parasite transmitted by the bite of a sand fly, which transmits an infectious metacyclic promastigote that subsequently transforms to an amastigote within mammalian macrophages. *Leishmania amazonensis* causes cutaneous leishmaniasis in tropical and subtropical countries of the Western hemisphere. *Leishmania* parasites have been shown to interfere with host cell functions, including the modulation of signaling pathways, suppression of antimicrobial and pro-inflammatory mediators, and induction of cytokines that promote disease progression.[1] Control of *L. amazonensis* within macrophages in vitro requires the production of reactive oxygen species and nitric oxide.[2, 3] Currently treatment is through the use of pentavalent antimonials.[4] Cutaneous and mucocutaneous leishmaniasis treatments are often poorly justified due to their limited effectiveness and numerous side effects. More research is needed to develop better treatment options with less severe side effects and better efficacy.

Autophagy plays a role in cell survival throughout health and disease including, aging, cancer, neurodegenerative diseases, immunity, infectious diseases, and chronic inflammatory conditions.[5] Canonical autophagy is an intracellular homeostatic mechanism important for the degradation of cytosomal components that range in size from single proteins to entire organelles via autodigestion through the lysosomal pathway. Multiple autophagic pathways exist in mammalian species and can be either nonselective or selective. Selective autophagy can target intracellular microorganisms in a process known

as xenophagy, and multiple host factors and pathways are activated and contribute to this process.[6] Autophagosomes can be recognized by their association with microtubule-associated protein 1 light chain 3 (LC3), which is conjugated with phosphotidylethanolamine (PE) to form LC3II. In canonical autophagy LC3II facilitates the formation of a double-membrane autophagosome; which fuses with a late endosome or lysosome to form the autolysosome.[7]

Recent studies have provided evidence for a role of autophagy in host cell defenses.[8] Autophagy has been shown to play a role in innate immunity and can be activated through numerous pattern recognition receptors to participate in the elimination of microorganisms. Other studies have also confirmed that autophagy can target intracellular parasites and bacteria, including *Listeria monocytogenes*, *Salmonella enterica*, *Francisella tularensis*, and *Toxoplasma gondii*.[9, 10, 11, 12, 13, 14, 15]

Non-canonical autophagy can be used by phagocytes to kill and digest pathogens.[16] Non-canonical autophagy is described as autophagy that is independent of some of the core canonical machinery components, such as the initiation factors ULK1 and ULK2.[6] Recently, a form of non-canonical autophagy has been described that utilizes LC3 recruitment and the fusion of the phagosome and lysosome without the formation of a double membrane autophagosome.[6, 16, 17] This type of non-canonical autophagy has been referred to as LC3-associated phagocytosis (LAP) and can be triggered by innate immune receptors such as Fc receptors, TLRs, or C-type lectin receptors.[16] LAP is similar to canonical autophagy with the utilization of Beclin-1 and class III PI3K activity

along with the recruitment of LC3 to the phagosome.[16] It is preceded by the generation of reactive oxygen species (ROS) through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which utilizes the protein Rubicon to further recruit LC3II to the phagosome membrane and induce LAP.[17, 18]

Inflammatory cytokines have also been involved in the activation of autophagy. IFN-γ may activate autophagy through immunity-related GTPases and through phosphorylation of beclin 1 by death-associated protein kinase 1 (DAPK1).[19] The signaling mechanisms that autophagy uses for the degradation of intracellular microorganisms are largely undetermined. More research is needed to elucidate the specific signaling pathways; however, it appears that lipid second messengers and phosphatidylinositol 3-phosphate (PI3P) are required for autophagy.[20] Shahnazari et al. have demonstrated that diacylglycerol (DAG) can serve as a signal to promote antibacterial autophagy via PKCδ. This leads to the activation of autophagy via NADPH oxidase pathways within phagocytes.[20]

Reactive oxygen species (ROS) are classical antimicrobial effectors, which play an important role in immune signaling. ROS produced by NADPH oxidase downstream of TLR or Fcγ receptor stimulation in phagocytes can activate non-canonical autophagy.[5, 18] We have previously shown that ROS are required to kill intracellular *Leishmania amazonensis.*[21] Numerous studies have shown a connection between ROS and non-canonical autophagy, although these signaling pathways are complex and not completely understood.[22]

In this report we show a mechanism for the reduction in *Leishmania amazonensis* infection through the activation of autophagy, as determined by the colocalization of LC3 with the parasite after stimulation of macrophages with soluble immune complexes, IFN-γ, and *Leishmania* antigen. Our lab has determined the necessary immune factors required for the reduction of *L. amazonensis* by comparing the limited, transient, infections of *L. major* to the chronic infections of *L. amazonensis*. We found that mice co-infected with both *L. major* and *L. amazonensis* would heal their infections. Throughout our studies we have determined that superoxide and nitric oxide are required to kill *L. amazonensis.* We have also determined that the production of superoxide can be achieved through the stimulation of macrophages by soluble immune complexes, IFN-γ, and *Leishmania* antigen.[23] We hypothesize that these three immune factors are providing receptor cooperation to stimulate non-canonical autophagy and removal of the parasite. These results are similar to the findings of Bezbradica et al, in which they found receptor cooperation between FcγRI and IFN-γR for the induction of the antimicrobial functions.[24] We found have found that activating macrophages with soluble immune complexes, IFN-γ, and *Leishmania* antigen, we not only see killing of the parasite; but we also find upregulation of autophagy which was dependent upon PKCδ. This is also similar to other studies that have shown a role of non-canonical autophagy in the clearance of intracellular microorganisms and its regulation by PKCδ.[9, 10, 11, 12, 13, 14, 15]

Materials and Methods

Mice

C3HeB/FeJ (C3H) mice (8-10 weeks of age) were obtained from an in-house breeding colony. Mice were maintained in a specific pathogen-free facility. Bone marrow from PKCδ-/- and C57BL/6 (B6) mice were obtained as a gift from Dr. A. Kanthasamy. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Bone marrow macrophages and cell culture

Bone marrow cells were harvested from femurs and tibias of C3H, B6 or PKCδ-/- animals (1-3 mice per experiment) and plated in 150 x 15 mm Petri dishes with 30ml of macrophage growth medium (30% L-cell conditioned medium, 20% fetal bovine serum (FBS), 50% Dulbecco's modification of eagle's medium (DMEM), 2mM L-glutamine, 100 U penicillin per ml, 100µg of streptomycin per ml and 1 mM sodium pyruvate) at 37°C and 5% CO_2, after 2 days an additional 20 ml of macrophage medium was added. At day 7, the adherent cell population was collected and, after washing with PBS, trypan blue exclusion was used to count live cells, which were resuspended in complete tissue culture medium (CTCM; DMEM, 2mM L-glutamine, 100 U penicillin, 100µg streptomycin/ml, 25 mM HEPES, 0.05 um 2-mercaptoethanol and 10% FBS).

RAW 264.7 Cells

RAW 264.7 mouse macrophage cells were maintained in complete tissue culture media (CTCM; DMEM, 2mM L-glutamine, 100 U penicillin, 100µg streptomycin/ml, 25 mM HEPES, 0.05 um 2-mercaptoethanol and 10% FBS).

Parasites and Antigens

L. amazonensis (MHOM/BR/00/LTB0016) promastigotes were grown in complete Grace's medium (Atlanta Biologicals, Lawrencville, GA) to stationary phase, harvested, washed in endotoxin free PBS (Cellgro, Herdon, VA) and prepared to a concentration of 1 x 10^8 parasites/mL. Freeze-thawed (FT) *Leishmania major*-antigen was obtained from stationary phase promastigotes as previously described.[25]

Macrophage infection and treatments

Bone marrow-derived macrophages (BMM) or RAW cells were infected with promastigotes as indicated in the figure legends at 3:1 parasite to cell ratio. In indicated experiments *L. amazonensis* promastigotes were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). The infected cells were incubated on coverslips at 34°C with 5% CO_2 in 24 well plates. After 24 hours, cells were washed with warm DMEM to remove extracellular parasites and brought to a final volume of 0.5 ml with CTCM.

Activation of infected macrophages using soluble immune complexes

Mouse IgG2a isotype (functional grade purified, eBioscience) was either unlabeled or labeled with Alexa Fluor (AF) 647 (Invitrogen), depending on experiment. Soluble immune complexes (ICs) were formed by combining mouse IgG2a and goat anti-mouse IgG F(ab')$_2$ (AffiniPure F(ab')$_2$ Fragment, Jackson ImmunoResearch) at a 2:1 molar ratio and incubated for 2 hours at 37°C with 5% CO_2. ICs were centrifuged at 15,000g for 10 minutes and the supernatant was collected for use.[26] Twenty-four hours following infection with *L. amazonensis*

promastigotes, macrophages were washed with warm DMEM and the media was replaced with 0.5 ml of fresh CTCM. Cells were then incubated with these pre-formed soluble ICs (10 µg/ml), IFN-γ (Peprotech, 5 ng/ml), FT-Ag (50 µg/ml), IgG2a (10 µg/ml), or $F(ab')_2$ (10 µg/ml), or combinations of these, as indicated. Cells were then incubated for 12 or 24 hours at 34°C with 5% CO_2. At the indicated timepoint, cells were harvested on coverslips and fixed with 4% paraformaldehyde for 10 minutes.

Determination of macrophage infection rate

Following incubation for 24 hours, coverslips were harvested, fixed and stained using nonspecific HEMA 3 stain set (Fisher Diagnostics, Middletown, VA). Coverslips were mounted onto glass slides and counted via light microscopy at 100x oil magnification. Three areas of 100 cells each were examined and the number of infected macrophages/100 cells was recorded.

Immunoblot Analysis

Protein content of all cell extracts was determined via nanodrop (ND-1000, Wilmington, Delaware), according to manufacturer's recommendations, and all samples were normalized to 2 mg/ml using distilled water. Samples (20 to 30 µg of protein) were heated for 4 minutes at 95°C in 6x SDS loading buffer and electrophoresis was performed on a 12% SDS-polyacrylamide electrophoresis gel. Gels were electroblotted onto polyvinylidene fluoride membranes, blocked with 5% dry milk, and probed with anti-LC3B antibody (ab48394, AbCam) or anti-Actin antibody (Sigma). Signals were detected with horseradish-peroxidase-conjugated goat anti-rabbit antibodies (1:10,000) (Sigma) using the SuperSignal

West chemiluminescent substrate (Pierce, Rockford, IL) and exposed to autoradiography film (Midsci, St. Louis, MO).

Determination of superoxide

Production of superoxide was assessed using CellROX (Invitrogen). CellROX was added to cell cultures at a final concentration of 5 μM after 24 hours and allowed to sit for 30 min (according to manufacturer's instructions). Coverslips were then analyzed via confocal microscopy. Coverslips were viewed by sequential scanning confocal microscopy using an Olympus IX81 inverted scope (Olympus America Inc., Center Valley, PA). Image J was used to determine the colocalization and intensity of signal of CellROX (superoxide) and parasite. Briefly, CFSE positive parasites were isolated and the intensity of Alexa Fluor 647 positive CellROX was measured in the isolated areas. An average of the CellROX intensity was measured taken after analysis of 5 images per each treatment group in each experiment.

Immunofluorescence

Following incubation for the designated times, coverslips with adherent RAWs were harvested and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and washed three times with PBS. RAWs were permeabilized with 1% Saponin in PBS for 30 minutes at room temperature. After incubation, coverslips were washed three times with PBS and incubated for 1 hour at room temperature with anti-LC3 conjugated antibody at a 1:200 dilution in 1% Saponin in PBS. RAWs were counterstained and mounted with Gold antifade 4'6-Diamidino-2-phenindole (DAPI) according to manufacturer's instructions (Life

Technologies, Grand Island, NY). Superoxide production was detected through the use of CellROX deep red reagent (Alexa Fluor 647) (Life Technologies, Grand Island, NY). CellROX was added to the cells for 30 minutes before fixation, according to manufacturer's instructions. Coverslips were viewed by sequential scanning confocal microscopy using an Olympus IX81 inverted scope (Olympus America Inc., Center Valley, PA).

Quantitative colocalization analysis was performed by counting LC3 and parasite association using Olympus Fluoview version 2.1c software. 150 parasites were counted; those that were in contact with an LC3 puncta were considered positive for colocalization, considering LC3 puncta that are involved with the parasitophorous vacuole may not co-localize with a parasite residing within the vacuole. Image J was used to determine the colocalization and intensity of signal of CellROX (superoxide) and parasite.

Results

Bone marrow derived macrophages infected with Leishmania amazonensis *and treated with tripartite activation upregulate LC3II.*

We have previously shown that BMM infected with *Leishmania amazonensis* can kill intracellular parasites after activation with soluble immune complexes, *Leishmania* antigen, and IFN-γ (tripartite activation)[23]. We were interested in the mechanisms of this model of parasite clearance and hypothesized that the autophagy pathway would be upregulated when macrophages are activated to kill intracellular parasites. Shanazari *et al* found the intracellular pathogen

(*Salmonella* Typhimurium) is targeted by autophagy in mammalian cells and dependent upon functional NADPH oxidase. Therefore, we wanted to determine if autophagy is also targeting intracellular *Leishmania* parasites in tripartite activated cells. We performed a western blot analysis for LC3II in BMM bone marrow derived macrophages infected with *Leishmania amazonensis* and treated 24 hours later with soluble IC, *Leishmania* antigen, and IFN-γ or the appropriate controls. Lysates were collected at day 4 post-treatment. We showed a significant increase in LC3II whenever soluble IC were added to *Leishmania amazonensis* infected macrophages. (Figure 1)

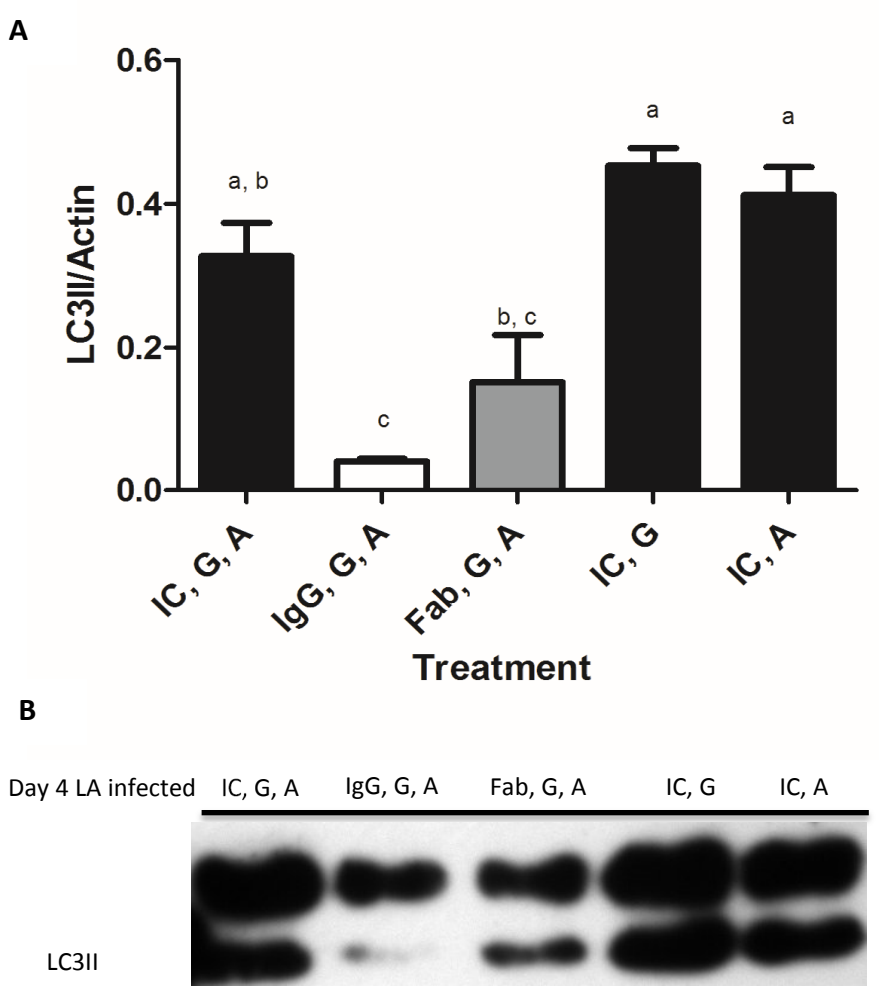

Figure 1. *Bone marrow derived macrophages infected with* Leishmania amazonensis (LA) *and activated with tripartite activation upregulate LC3II.* A: Bone marrow derived macrophages were infected with LA promastigotes and activated 24 hours later for 4 days. Treatments included soluble immune complexes (IC) (10 µg/ml) with IFN- γ (5 ng/ml) and *Leishmania* antigen (50 µg/ml); IgG2a (5 µg/ml) with IFN- γ (5 ng/ml) and *Leishmania* antigen (50 µg/ml); $F(ab)_2$ (5 µg/ml) with IFN- γ (5 ng/ml) and *Leishmania* antigen (50 µg/ml); IC (10 µg/ml) with IFN- γ (5 ng/ml); and IC (10 µg/ml) with *Leishmania* antigen (50 µg/ml) . Lysates were collected and immunofluorescence was completed for LC3II and Actin. In bone marrow macrophages there is an upregulation of LC3 in LA infected macrophages whenever soluble immune complexes are added. Results are densitometry of 5 separate experiments. B: Image from one representative experiment.

RAW 264.7 macrophages infected with Leishmania amazonensis *and treated with tripartite activation show a decrease in parasite load at 24 hours post treatment*

We have previously shown that this tripartite activation system in bone marrow derived macrophages after four days of treatment.[23] Next, we wanted to study this system in vitro in RAW 264.7 macrophages as they are commonly used in autophagy studies and it is known that they produce low levels of NOX2-generated ROS for a short duration compared to bone marrow derived macrophages.[27, 28] We showed that RAW 264.7 cells had a decrease in parasite load at 24 hours post-activation (Figure 2), compared to four days post activation in bone marrow derived cells. Confirming that tripartite activation recapitulates the results of bone marrow derived macrophages. However, a western blot for LC3II in treated RAW 264.7 cell lysates infected with *Leishmania amazonensis* showed no significant increase in LC3II protein expression compared to controls (data not shown).

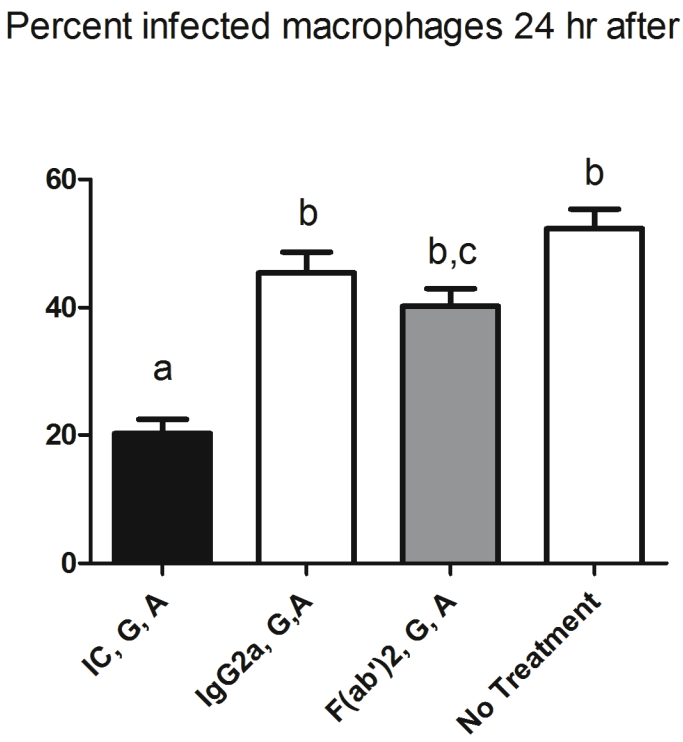

Figure 2. *RAW 264.7 macrophages infected with* Leishmania amazonensis *and treated with tripartite activation show a decrease in parasite load at 24 hours post treatment*
RAW 264.7 macrophages were infected with LA promastigotes for 24 hours then treated for 24 hours (as in Figure 1) until coverslips were collected, fixed and mounted for counting infected via light microscopy. Three areas of 100 macrophages were counted under 100X oil immersion and the number of macrophages containing intracellular LA was recorded. The results include three separate experiments. a = $P<0.0001$.

Tripartite activation of infected RAW 264.7 cells leads to colocalization of LC3II with Leishmania amazonensis

Due to the lack of upregulation of LC3II in RAW 264.7 cells we focused on the distribution of LC3II in infected and activated cells. We hypothesized that the location of LC3II was an important readout as to regulation of autophagy during parasite killing post-activation. RAW 264.7 cells infected with *Leishmania*

amazonensis and treated with the tripartite activation (soluble immune complexes, *Leishmania*-antigen, and IFN-γ) had a significant increase in parasite and LC3II colocalization as compared to the controls (Figure 3). The addition of IgG2a or $F(ab)_2$ alone with *Leishmania*-antigen, and IFN-γ was not enough to see colocalization.

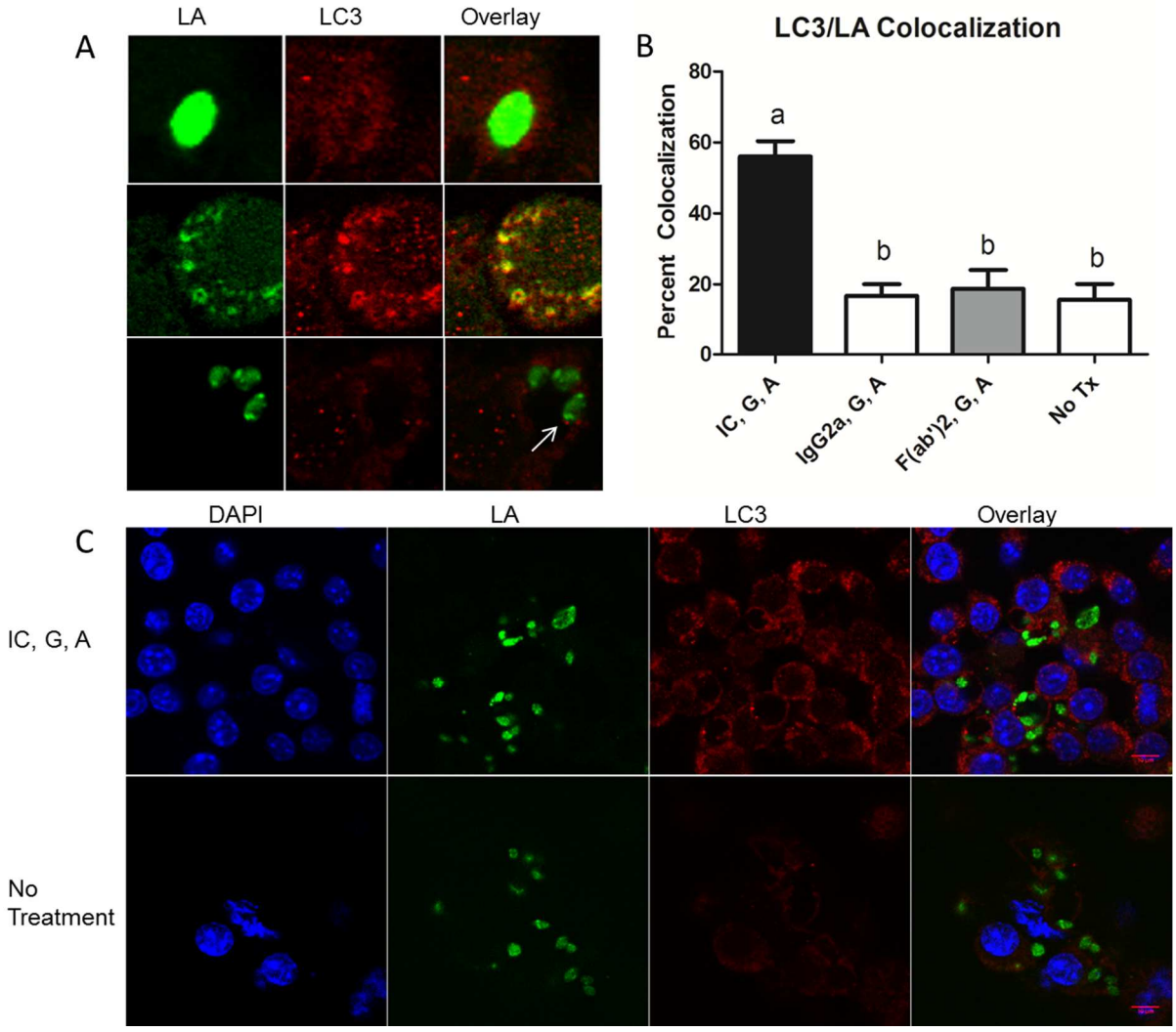

Figure 3. *Tripartite activation of infected RAW 264.7 cells leads to colocalization of LC3II with Leishmania amazonensis.*
RAW 264.7 macrophages were infected with LA promastigotes for 24 hours then treated for 24 hours as in Figure 1, until coverslips were collected, fixed and mounted for confocal microscopy. LC3/LA colocalization was counted using Olympus Fluoview version 2.1c software. Colocalization of 150 parasites with

LC3 were counted; those that were in contact or overlapping with an LC3 puncta were considered positive for colocalization. A. Examples of positive and negative colocalization. Top row: negative colocalization; Middle row: positive colocalization; Bottom row: Positive colocalization (arrow), others are negative. B. There was a significant increase in LC3/LA colocalization in cells treated with soluble IC, IFN-γ, and *Leishmania* antigen. Results include three separate experiments. P=<0.001 C. Tripartite activation shows positive parasite and LC3 colocalization compared to no treatment which shows no parasite and LC3 colocalization. The results are pooled from two separate experiments in duplicate.

Tripartite activation of infected RAW 264.7 cells leads to an increase in the intensity of superoxide associated with Leishmania amazonensis

Our lab has shown in previous work that both superoxide and nitric oxide are necessary to kill intracellular *Leishmania amazonensis*.[21] Therefore, we were interested in determining if our system was activating superoxide production near the intracellular parasites. We used a CellROX analysis to detect superoxide production via confocal microscopy. RAW 264.7 cells were infected with CFSE-labeled *Leishmania amazonensis* and treated the following day with soluble immune complexes, *Leishmania*-antigen, and IFN-γ or the appropriate controls. At 24 hours post-treatment CellROX was added for 30 minutes and the cells were fixed and mounted for confocal microscopy. There was a significant increase in the intensity of superoxide overlying the parasite when the cells were treated with tripartite activation as compared to the controls. Again, this was only true when all 3 activation molecules were added to the treatment and it did not occur with the addition of IgG2a or $F(ab)_2$ with both *Leishmania*-antigen, and IFN-γ.

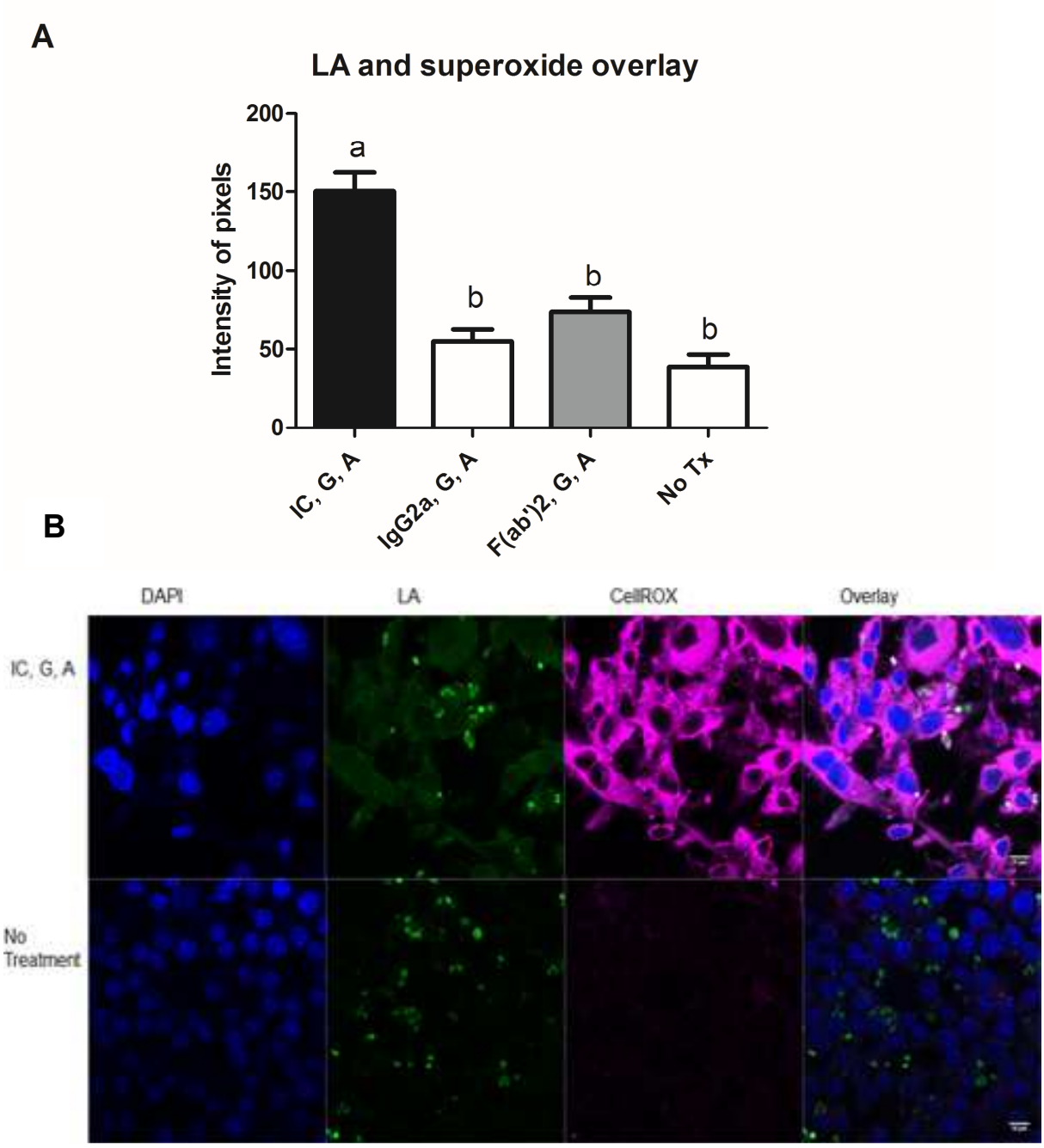

Figure 4. *Tripartite activation of infected RAW 264.7 cells leads to an increase in the intensity of superoxide overlying* Leishmania amazonensis.
RAW 264.7 macrophages were infected with LA promastigotes for 24 hours then treated for 24 hours until coverslips were collected, fixed and mounted for confocal microscopy. Image J was used to determine the colocalization of CellROX (superoxide) and parasite. CFSE positive parasites were isolated and the intensity of Alexa Fluor 647 positive CellROX was measured in the isolated areas. An average of the CellROX intensity was measured after analysis of 5 images per treatment group in each experiment. There was a significant increase

in CellROX intensity overlying LA in cells treated with soluble IC, IFN-γ, and Leishmania antigen. Results are a total of two separate experiments. B. Tripartite activation showed a dectectable increase in CellROX intensity overlying parasites compared to no treatment.

Protein kinase C delta (PKC δ) is required for killing of Leishmania amazonensis*, LC3II and parasite colocalization, and superoxide production*

Shahnazari et al. found that diacyclglycerol can promote autophagy by recruiting PKC*δ*, which can activate autophagy via c-Jun N-terminal kinase (JNK) and NADPH oxidase pathways.[20] Due to the similarities to *Salmonella* infection, we hypothesized that this signaling pathway was also playing a significant role in our system and that inhibiting this pathway would inhibit the activities of the tripartite activation. Bone marrow macrophages were derived from PKC*δ-/-* on a C57BL/6 background along with macrophages from wild-type C57BL/6 as controls. The macrophages were infected with *Leishmania amazonensis* promastigotes and 24 hours later treated with tripartite activation. After 4 days of treatment, cells were fixed and analyzed either by light microscope for parasite counts or confocal microscopy for LC3 and CellROX localization and production, respectively. PKC*δ-/-* macrophages did not have a decrease in parasite load as compared to the wild-type C57Bl/6 controls. Additionally, the PKC*δ-/-* BMM did not show colocalization of LC3II and parasites and did not have an increase in superoxide production associated with parasites as compared to the wild-type C57Bl/6 controls. These results demonstrate that this pathway is integral to the autophagy

and NADPH pathways in the clearance of *Leishmania amazonensis* following tripartite activation.

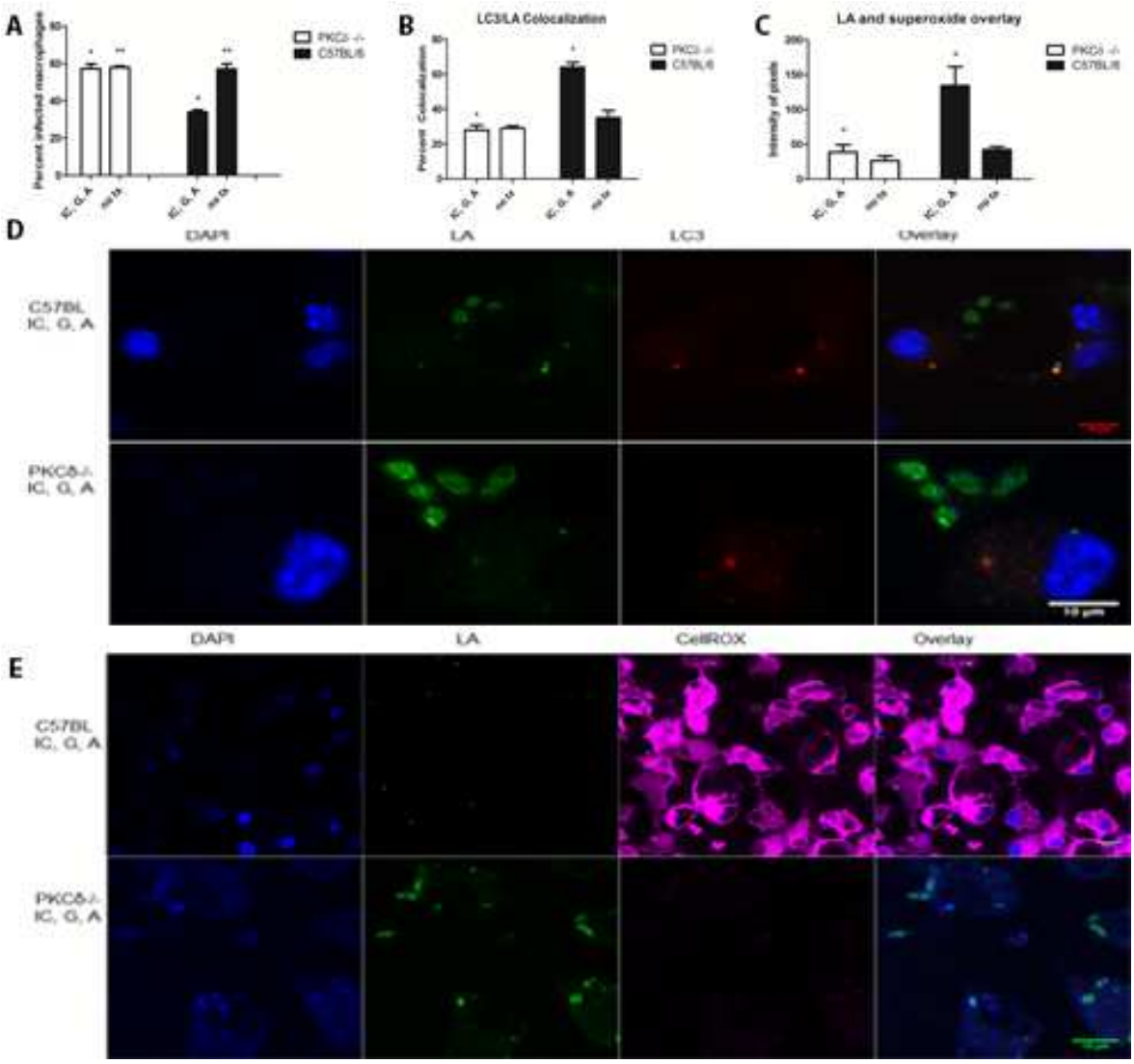

Figure 5. *Protein kinase delta C (PKC-δ) deficient (-/-) bone marrow derived macrophages are unable to kill intracellular Leishmania amazonensis, do not promote LC3II and parasite colocalization, and do not produce superoxide.* C57Bl/6 wildtype and PKC δ -/- bone marrow derived macrophages were infected with LA promastigotes and treated 24 hours later for 4 days as in Figure 1. A and B. Coverslips were collected, fixed and mounted for counting infected macrophages via light microscopy. LC3/LA colocalization was determined using Olympus Fluoview version 2.1c as previously described. C. Coverslips were collected, fixed and mounted for confocal microscopy. Image J was used to determine the colocalization of CellROX (superoxide) and parasite as previously

described. Results are a total of two separate experiments in duplicate. D. Wild-type and PKCδ -/- cells treated with tripartite activation and processed and imaged as in A. E. Wild-type PKCδ -/- cells treated with tripartite activation and processed as in C.

Discussion

Our results show that IFN-γ, soluble immune complexes, and *Leishmania*-antigen are all required to trigger an antimicrobial response against *Leishmania amazonensis*.

Only all three immune factors triggered an antimicrobial response with a corresponding increase in superoxide production and colocalization of LC3 to the parasite. A recent article has described the structural and functional cooperation between the IFN-γ and FcγRI receptors; allowing these receptors to act as a coincidence detection system eliciting unique gene-expression programs when both receptors are engaged.[24] Only the activation of both receptors triggered the induction of antimicrobial functions against *L. amazonensis*. This coincidence detection system allows a distinct response when both signals are present; however, the extent of dependence on signals from other receptors could vary in different situations.[24] Here we describe the need for cooperation between the activation of three potential receptors in order to induce antimicrobial functions against *L. amazonensis*.

We show that these effector molecules act through the PKCδ pathways to activate autophagy via the NADPH oxidase pathway with colocalization of LC3II with the parasite. This pathway is consistent with a recent article that has described the promotion of autophagy of *Salmonella* Typhimurium through these

pathways.[20] This is also similar to the findings by Shahnazari et al. that demonstrate a diacylglycerol-dependent signaling pathway in the regulation of antibacterial autophagy.[29, 30] Shahnazari et al. propose that DAG acts at the target organelle to recruit PKC*δ,* which can activate autophagy via the JNK and NADPH oxidase pathways.[20] We found that the loss of PKC*δ* through the use of knock out mice resulted in a loss of the antimicrobial actions of the macrophages along with a decrease in LC3 colocalization and superoxide production.

We found that the addition of soluble immune complexes to bone marrow derived macrophages lead to an increase in LC3II. We also found that the autophagy pathway can play a role in the clearance of *Leishmania amazonensis* in infected macrophages and PKC*δ* plays an important role in autophagy signaling. We have previously shown that soluble ICs can promote parasite clearance through the generation of antibody-dependent superoxide production.[23] The assembly of NADPH oxidase can eventually lead to the recruitment of molecules integral to the autophagy pathway and activation of this pathway.[27] The activation of antibacterial autophagy by NADPH oxidases has been described by Huang et al, which can be extrapolated to other intracellular pathogens, such as *Leishmania*.[27]

The immunomodulation of these pathways may lead to potential therapeutic benefits for treatment of intracellular pathogens, especially *Leishmania* species. The ability to target the microbe to autophagy pathways without the use of antimicrobials could be a potential route to decrease antimicrobial resistance amongst pathogens. Here we have described how a new

mechanism by which antibodies function to clear an infection via NADPH oxidase and autophagy pathways.[31] Further research is needed to elucidate the specific signaling pathways such as to determine specific signals involved in FcγR mediated phagocytosis. Specific signaling and receptor cooperation that is involved in the upregulation of autophagy for the clearance of *Leishmania amazonensis* also needs to be elucidated. Finally, determining the specific molecules that are involved in the anti-*Leishmania* autophagy pathway will be integral in specifying the type of non-canonical autophagy that is occurring and the formation of LAPosomes.

References

1. Boggiatto, P.M. *et al.* Altered Dendritic Cell Phenotype in Response to Leishmania amazonensis Amastigote Infection Is Mediated by MAP Kinase, ERK. *American Journal of Pathology* **174**, 1818-1826 (2009).

2. Mukbel, R., Petersen, C.A. & Jones, D.E. Soluble factors from Leishmania major-specific CD4(+)T cells and B cells limit L-amazonensis amastigote survival within infected macrophages. *Microbes and Infection* **8**, 2547-2555 (2006).

3. Mukbel, R.M. *et al.* Macrophage killing of Leishmania amazonensis amastigotes requires both nitric oxide and superoxide. *American Journal of Tropical Medicine and Hygiene* **76**, 669-675 (2007).

4. Monge-Maillo, B. & Lopez-Velez, R. Therapeutic options for old world cutaneous leishmaniasis and new world cutaneous and mucocutaneous leishmaniasis. *Drugs* **73**, 1889-1920 (2013).

5. Deretic, V. & Levine, B. Autophagy, Immunity, and Microbial Adaptations. *Cell Host & Microbe* **5**, 527-549 (2009).

6. Huang, J. & Brumell, J.H. Bacteria-autophagy interplay: a battle for survival. *Nature Reviews Microbiology* **12**, 101-114 (2014).

7. Simon, D.J.P.a.A.K. Autophagy in the immune system. *Immunology*, 1-8 (2013).

8. Birmingham, C.L., Smith, A.C., Bakowski, M.A., Yoshimori, T. & Brumell, J.H. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *Journal of Biological Chemistry* **281**, 11374-11383 (2006).

9. Byfield, M.P., Murray, J.T. & Backer, J.M. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *Journal of Biological Chemistry* **280**, 33076-33082 (2005).

10. Brazil, M.I., Weiss, S. & Stockinger, B. Excessive degradation of intracellular protein in macrophages prevents presentation in the context of major histocompatibility complex class II molecules. *European Journal of Immunology* **27**, 1506-1514 (1997).

11. Massey, A.C., Zhang, C. & Cuervo, A.M. Chaperone-mediated autophagy in aging and disease. *Current Topics in Developmental Biology, Vol 73* **73**, 205-+ (2006).

12. Lum, J.J., DeBerardinis, R.J. & Thompson, C.B. Autophagy in metazoans: Cell survival in the land of plenty. *Nature Reviews Molecular Cell Biology* **6**, 439-448 (2005).

13. Meijer, A.J. & Codogno, P. Regulation and role of autophagy in mammalian cells. *International Journal of Biochemistry & Cell Biology* **36**, 2445-2462 (2004).

14. Maderna, P. & Godson, C. Phagocytosis of apoptotic cells and the resolution of inflammation. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **1639**, 141-151 (2003).

15. Lund, J., Sato, A., Akira, S., Medzhitov, R. & Iwasaki, A. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. *Journal of Experimental Medicine* **198**, 513-520 (2003).

16. Mehta, P., Henault, J., Kolbeck, R. & Sanjuan, M.A. Noncanonical autophagy: one small step for LC3, one giant leap for immunity. *Curr Opin Immunol* **26**, 69-75 (2014).

17. Martinez, J. *et al.* Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. *Nat Cell Biol* **17**, 893-906 (2015).

18. Yang, C.S. *et al.* Autophagy Protein Rubicon Mediates Phagocytic NADPH Oxidase Activation in Response to Microbial Infection or TLR Stimulation. *Cell Host & Microbe* **11**, 264-276 (2012).

19. Gutierrez, M.G. *et al.* Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766 (2004).

20. Shahab Shahnazari, W.-L.Y., Cheryl L. Birmingham, Jessica Shiu, Anton Namolovan, Yiyu T. Zheng, Keiko Nakayama, Daniel J. Klionsky, and John H. Brumell. A Diacyclglycerol-Dependent Signaling Pathway Contributes to Regulation of Antibacterial Autophagy. *Cell Host and Microbe*, 137-146 (2010).

21. Mukbel, R.M. *et al.* Macrophage killing of Leishmania amazonensis amastigotes requires both nitric oxide and superoxide. *The American journal of tropical medicine and hygiene* **76**, 669-675 (2007).

22. Deretic, V., Saitoh, T. & Akira, S. Autophagy in infection, inflammation and immunity. *Nature reviews. Immunology* **13**, 722-737 (2013).

23. Gibson-Corley, K.N. *et al.* An In Vitro Model of Antibody-Enhanced Killing of the Intracellular Parasite Leishmania amazonensis. *PLoS One* **9**, e106426 (2014).

24. Jelena S Bezbradica, R.K.R., Richard A DeMarco, Igor Brodsky, Ruslan Medzhitov. A role for the ITAM signaling molecule in specifying cytokine-receptor functions. *Nature Immunology* **15**, 333-345 (2014).

25. Jones, D.E., Ackermann, M.R., Wille, U., Hunter, C.A. & Scott, P. Early enhanced Th1 response after Leishmania amazonensis infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infection and immunity* **70**, 2151-2158 (2002).

26. Nimmerjahn, F. & Ravetch, J.V. Analyzing antibody-Fc-receptor interactions. *Methods Mol Biol* **415**, 151-162 (2008).

27. Huang, J. *et al.* Activation of antibacterial autophagy by NADPH oxidases. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 6226-6231 (2009).

28. Mantegazza, A.R. *et al.* NADPH oxidase controls phagosomal pH and antigen cross-presentation in human dendritic cells. *Blood* **112**, 4712-4722 (2008).

29. Shahnazari, S., Namolovan, A., Klionsky, D.J. & Brumell, J.H. A role for diacylglycerol in antibacterial autophagy. *Autophagy* **7**, 331-333 (2011).

30. Shahnazari, S. *et al.* A Diacylglycerol-Dependent Signaling Pathway Contributes to Regulation of Antibacterial Autophagy. *Cell Host & Microbe* **8**, 137-146 (2010).

31. Gibson-Corley, K.N. *et al.* An In Vitro Model of Antibody-Enhanced Killing of the Intracellular Parasite Leishmania amazonensis. *Plos One* **9** (2014).

CHAPTER 4

CROSSLINKING OF FCγR WITH A RECOMBINANT FC CONSTRUCT CAN ACTIVATE MACROPHAGES TO PRODUCE SUPEROXIDE AND ENHANCE INTRACELLULAR KILLING OF *LEISHMANIA amazonensis*

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Abstract

We have previously shown that macrophage activation in response to soluble IgG2a immune complexes, IFN-γ, and parasite antigen was effective in killing *Leishmania amazonensis;* and that intracellular killing of *L. amazonensis* parasites is dependent upon FcRγ common-chain and NADPH oxidase-generated superoxide from infected macrophages. Here we show that activation of a macrophage with a novel recombinant murine IgG2a-Fc construct will result in construct trafficking and induce superoxide production. Preliminary studies also show killing of *L. amazonensis*. A recombinant murine IgG2a-Fc construct was created by expressing the protein in HEK293F cells. This construct has homologous morphology to the Fc portion of IgG2a consisting of the Fc portion of a single heavy chain, which naturally forms a dimer with another heavy chain, creating a single homodimer of Fc fragment of IgG2a. These experiments define a new mechanism by which recombinant constructs can be developed to manipulate the immune response to promote killing of an intracellular pathogen, post-infection.

Introduction

Cutaneous leishmaniasis can be caused by the intracellular protozoan parasite *Leishmania amazonensis*. Leishmaniasis is endemic in many tropical and subtropical countries, and is currently considered a neglected tropical disease. Cutaneous leishmaniasis can lead to severe disease that begins as an insect bite that gradually enlarges and can become an open sore with occasional disfiguration. Spontaneous resolution generally occurs, although the time to resolution varies.[1] Clearance of *L. amazonensis* requires the production of reactive oxygen species and nitric oxide.[2] There are no available vaccines for cutaneous leishmaniasis and most treatments have limited effectiveness with numerous side effects.

Our lab has previously shown that reduction of *L. amazonensis* is parasites in vitro can be dependent upon Fragment crystallizable receptor-γ (FcRγ) common-chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase generated superoxide, and iNOS generated nitric oxide from infected macrophages. We discovered a method of macrophage activation with non-specific soluble IgG2a immune complexes, IFN-γ, and parasite antigen that were effective in killing *L. amazonensis*. We have previously found that soluble immune complexes promoted a NADPH oxidase-dependent leishmanicidal response post-infection.[3] This is a novel means by which a host can effectively use IgG antibodies to effectively enhance killing of intracellular pathogens.

Here we describe the production of a novel recombinant protein that has homology with the Fc portion of murine IgG2a. This protein was designed to

replace the use of non-specific soluble immune complexes in our system for activating macrophages. The production of recombinant proteins allows for a high yield of proteins that can be tagged with GFP or other proteins. These proteins can also be labeled with different glycosylation patterns, which can affect their affinity for their receptor.[4]

Immunoglobulin engagement of antibody Fc receptors, which are found on many cell surfaces, can induce a variety of immune functions by regulating intracellular signaling. Antibody Fc receptors are named by their ability to bind a specific immunoglobulin isotype. FcγRI (CD64) is present on the surface of monocytes and macrophages, and bind, with high affinity, the Fc portion of IgG1 or IgG2a in humans and mice, respectively. The main immune functions of Fc receptor engagement are facilitation of phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), induction of release of inflammatory mediators, and regulation of lymphocyte proliferation and differentiation.[5] Given their wide array of functions in modulating the immune response new and promising strategies have unfolded for developing molecules that are able to mimic FcR-Ig interaction to exploit immunoregulation.[6]

Cell surface expression of FcγRI can be induced by IFNγ. FcγRI in turn can bind monomeric IgG2a and take it up via endocytosis in mice.[7] Cross-linked receptors lead to internalization of the antigen-antibody complex and activation of the cell to produce effector functions, such as superoxide production.[7] FcγRI signals through a common γ chain and activates the proto-oncogene tyrosine protein kinase (SRC) family of kinases, spleen tyrosine kinase (SYK), and has been

shown to induce superoxide production when bound by large insoluble IC.[8] However, when small soluble immune complexes (ICs) bind the FcγRI, they are internalized by endocytic pathways, leading to other functions, such as receptor expression, regulation of signal transduction, antigen presentation, endosome recycling, and other actions.[9] Other studies have shown that these small soluble ICs can also induce superoxide production upon cross-linking of the FcγRI.[10, 11] The ability to manipulate these receptors in order to get a timely and localized release of inflammatory mediators can be helpful in inducing an effective immune response against intracellular pathogens. In this study we have found that the production of novel recombinant Fc constructs can bind and activate macrophages to kill intracellular *Leishmania amazonensis* and induce superoxide production.

Materials and Methods

Mice

C3HeB/FeJ (C3H) mice (8-10 weeks of age) were obtained from an in-house breeding colony. Mice were maintained in a specific pathogen free facility. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Bone marrow macrophages and cell culture

Bone marrow cells were harvested from femurs and tibias of C3H mice and plated in 150 x 15 mm Petri dishes with 30 ml of macrophage medium (30% L-cell conditioned medium, 20% fetal bovine serum (FBS), 50% Dulbecco's

modification of eagle's medium (DMEM), 2 mM L-glutamine, 100 U penicillin per ml, 100 µg of streptomycin per ml, and 1 mM sodium pyruvate) at 37° C and 5% CO_2, after 2 days an additional 20 ml of macrophage medium was added. At day 7, the adherent cell population was collected and, after washing with PBS, trypan blue exclusion was used to count live cells, which were resuspended in complete culture medium (CTCM; DMEM, 2 mM L-glutamine, 100 U penicillin, 100 µg streptomycin per ml, 25 mM HEPES, 0.05 µm 2-mercaptoethanol, and 10% FBS).

J774 and RAW264.7 Cells

The RAW264.7 and J774 mouse macrophage cell lines were maintained in complete tissue culture media (CTCM; DMEM, 2 mM L-glutamine, 100 U penicillin, 100 µg streptomycin per ml, 25 mM HEPES, 0.05 µm 2-mercaptoethanol, and 10% FBS).

Parasites and Antigen

L. amazonensis (MHOM/BR/00/LTB0016) promastigotes were grown in complete Grace's medium (Atlanta Biologicals, Lawrenceville, GA) to stationary phase, harvested, washed in endotoxin free PBS (Cellgro, Herdon, VA) and prepared to a concentration of 1 x 10^8 parasites per ml. Freeze-thawed *Leishmania major* antigen was obtained from stationary phase promastigotes as previously described.[2]

Transfection of HEK293 cells for the production of the recombinant Fc construct

HEK293 cell cultures are maintained in Medium A and Medium B at 37°C. Approximately 1 x 10^6 cells per ml are incubated with purified pGEn2 vector

encoding the target plasmid DNA for 24 hours. After an additional 4-5 days of incubation, cells are harvested for protein purification. The protein is purified using protein-A sepharose column and analyzed by SDS-PAGE.

Macrophage infection and treatments

RAWs were infected with promastigotes as indicated in the figure legends at 3:1 parasites to cell ratio. In indicated experiments *L. amazonensis* promastigotes were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). The infected cells were incubated on coverslips at 34ºC with 5% CO_2 in 24 well plates. After 24 hours, macrophages were washed with DMEM to remove extracellular parasites and brought to a final volume of 0.5 ml with CTCM.

Activation of infected macrophages using soluble immune complexes or Fc construct

Mouse IgG2a (functional grade purified, eBioscience) was either unlabeled or labeled with Alexa Fluor (AF) 647 (Invitrogen). Soluble immune complexes (ICs) were formed by combining mouse IgG2a and goat anti-mouse IgG $F(ab')_2$ (AffiniPure $F(ab')_2$ Fragment, Jackson ImmunoResearch) at a 2:1 molar ratio and incubated for 2 hours at 37ºC with 5% CO_2. ICs were centrifuged at 15,000g for 10 minutes and the supernatant was collected for use.[3] Twenty-four hours following infection with *L. amazonensis* promastigotes, macrophages were washed with warm DMEM and the media was replaced with 0.5 ml CTCM. Cells were then incubated with these pre-formed soluble ICs (10 µg/ml), IFN-γ (5 ng/ml, Peprotech), FT-Ag (50 µg/ml), IgG2a (10 µg/ml), or $F(ab')_2$ (10 µg/ml), or combinations of these, as indicated. Cells were then incubated for 12 or 24 hours

at 34°C with 5% CO_2. At the indicated timepoint, cells were harvested on coverslips and fixed with 4% paraformaldehyde for 10 minutes.

Determination of macrophage infection rate

Following incubation for 24 hours, coverslips were harvested, fixed and stained using nonspecific HEMA 3 stain set (Fisher Diagnostics, Middletown, VA). Coverslips were mounted onto glass slides and counted via light microscopy at 100x oil magnification. Three areas of 100 cells each were examined and the number of infected macrophages/100 cells was recorded.

Binding and inhibition of binding

Fc construct or IgG2a were added to cell cultures for a given amount of time, as indicated, then fixed for immunofluorescence with 4% paraformaldehyde for 10 minutes and washed three times with 1X phosphate buffered saline (PBS). After coverslips were washed they were mounted with Gold antifade 4'6-Diamidino-2-phenindole (DAPI) according to manufacturer's instructions (Life Technologies, Grand Island, NY). Binding inhibition study was done by adding Fc construct or IgG2a for 30 minutes, then macrophages were washed with endotoxin free PBS and the opposing molecule was added to the cells for 10 minutes. The cells were then fixed and visualized with confocal microscopy as previously described.

Endocytosis and phagocytosis inhibition

RAWs were incubated for 1 hour at 37°C with 80 μM Dynasore, 0.2% DMSO, or 5 μg/ml of Cytochalasin D in DMEM. After 1 hour, Fc-coated bead, Fc construct, along with IFN-γ were added to the appropriate wells. Cells were incubated for 0, 0.5, 1, 2, 4, and 12 hours then fixed with 4% paraformaldehyde and visualized

with confocal microscopy.

Determination of superoxide

Production of superoxide was assessed using CellROX (Invitrogen). CellROX was added to cell cultures after 24 hours and allowed to incubate at 37°C for 30 min (according to manufacturer's instructions). Coverslips were then analyzed via confocal microscopy.

Immunofluorescence

Following incubation for the designated times, coverslips with adherent RAWs were harvested and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and washed three times with PBS. RAWs were permeabilized with 1% Saponin in PBS for 30 minutes at room temperature. After incubation, coverslips were washed three times with PBS and counterstained and mounted with Gold antifade 4'6-Diamidino-2-phenindole (DAPI) according to manufacturer's instructions (Life Technologies, Grand Island, NY). Superoxide production was detected through the use of CellROX deep red reagent (Life Technologies, Grand Island, NY) as described above. Coverslips were viewed by sequential scanning confocal microscopy using an Olympus IX81 inverted scope (Olympus America Inc., Center Valley, PA). Image J was used to determine the colocalization of CellROX (superoxide) and parasite. Briefly, CFSE positive parasites were isolated and the intensity of CellROX was measured in these isolated areas. An average of the CellROX intensity was measured after analysis of 5 images per each treatment group in each experiment.

Results

Murine IgG2a Fc construct developed in HEK293F cells

In our in vitro disease model, we are able to kill intracellular *L. amazonensis* by activation of macrophages with soluble immune complexes of IgG2a isotype, IFN- γ, and *Leishmania* antigen. We hypothesized that the production of a homologous Fc construct protein would act in a similar manner when compared to murine IgG2a. Fc construct was produced in HEK293F cells as described in materials and methods and purified and tested as a replacement for nonspecific soluble IgG2a immune complexes in our previously described tripartite activation of macrophages.[3]

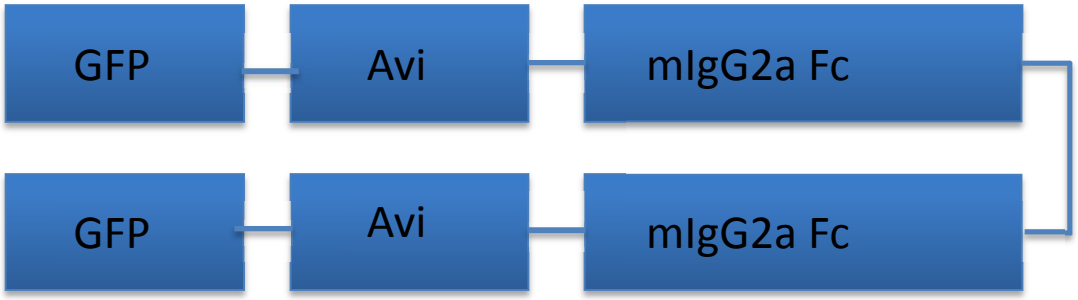

Figure 1. Murine IgG2a Fc construct developed in HEK293F cells. The construct is tagged with GFP and Avi for biotin binding and naturally forms a dimer.

Fc construct binds to murine macrophages

Murine macrophages incubated with GFP-tagged Fc construct had similar staining pattern compared to macrophages incubated with AF 647 labeled murine IgG2a. GFP-Fc construct and AF647-IgG2a can be seen along the cell membrane and often within the cytoplasm of the murine macrophages (Figure 2). Bone marrow derived macrophages and J774 macrophages had similar results with RAWs (data not shown).

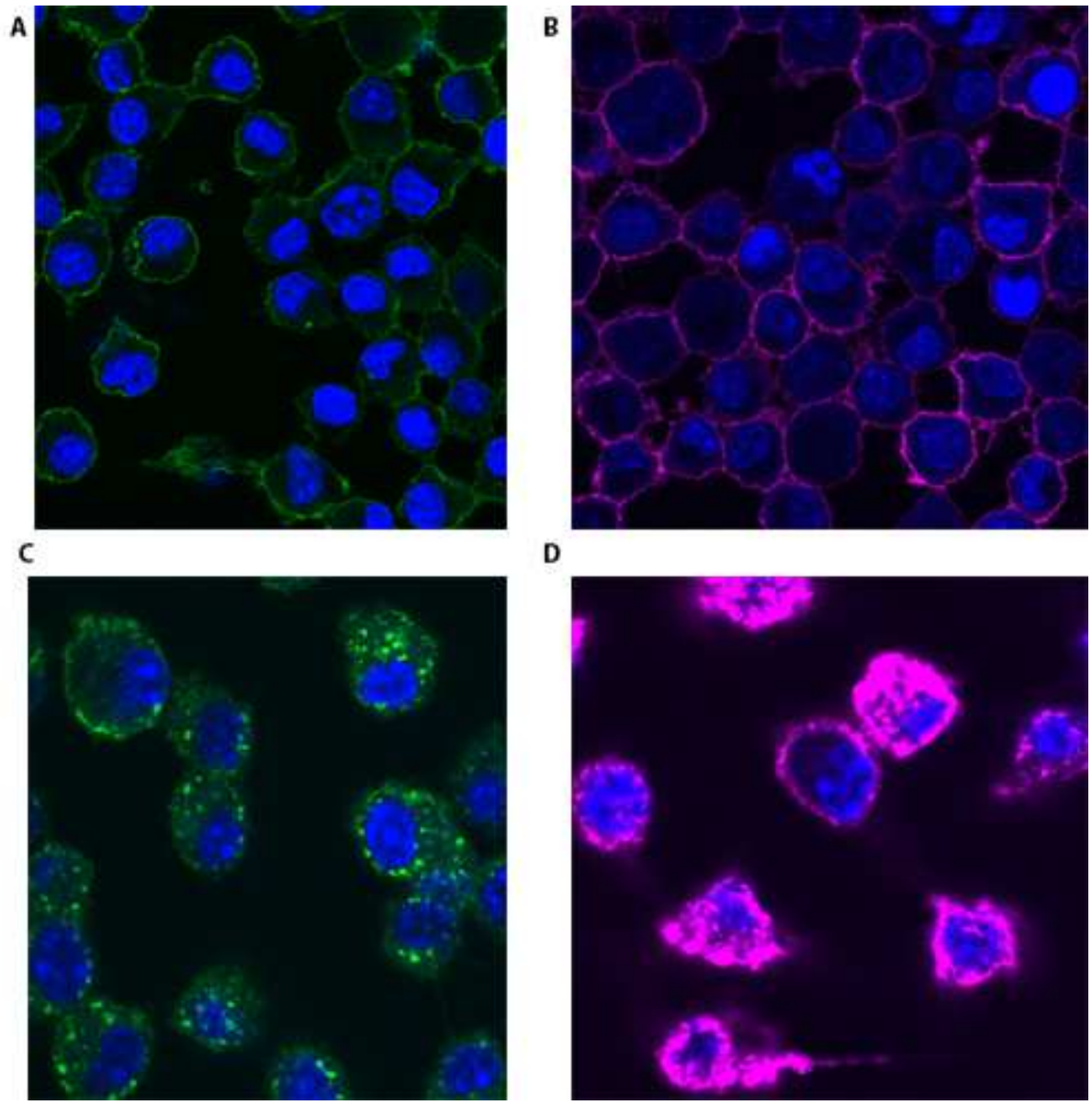

Figure 2. Murine macrophages incubated with GFP-tagged recombinant IgG2a Fc construct or with Alexa Fluor 647 labeled IgG2a. A and B: J774 cells incubated with Fc (5 µg/ml) (A) or IgG2a (5 µg/ml) (B) for 1 minute. C and D: Murine bone marrow derived macrophages incubated for 30 minutes with Fc (C) or IgG2a (D). Nuclei were stained with Dapi. Images are representative of three separate experiments in duplicate.

Fc construct inhibits binding of IgG2a to murine macrophages

To test the hypothesis that both the Fc construct and IgG2a were engaging the same Fc receptors, we performed an inhibition of binding assay. Macrophages incubated with Fc construct blocked the binding of IgG2a to the cells and IgG2a blocked the Fc construct from binding to the macrophages (Figure 3). Therefore, we concluded that these proteins were both binding to the same Fc receptors.

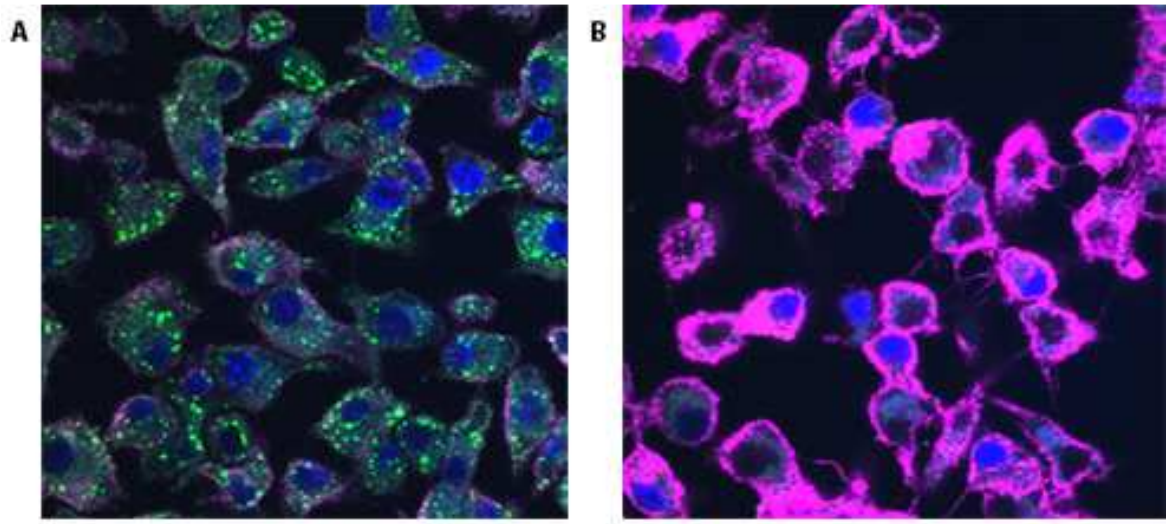

Figure 3. Recombinant IgG2a Fc construct inhibits binding of AF647 labeled IgG2a and vice versa. A: Murine bone marrow derived macrophages were incubated for 30 minutes with recombinant IgG2a Fc construct (GFP-tagged) (5 µg/ml) and incubated for 10 minutes with AF647 labeled IgG2a (5 µg/ml). B: Murine bone marrow derived macrophages were incubated for 30 minutes with Alexa Fluor 647 labeled IgG2a (5 µg/ml) then washed and incubated for 10 minutes with recombinant IgG2a Fc construct (GFP-tagged) (5 µg/ml). Nuclei were stained with Dapi. Images are representative of three separate experiments in duplicate.

Fc construct activation of RAW264.7 macrophages kills intracellular L. amazonensis *post-infection*

Previously we have shown that activation of macrophages with soluble immune complexes of the IgG2a isotype, IFN- γ, and *Leishmania* antigen can kill the

intracellular parasite. This occurs through the production of superoxide, which is required to kill *L. amazonensis*.[3] We hypothesized that soluble immune complexes were activating FcRγI leading to the production of superoxide. Since the Fc construct and murine IgG2a have similar morphology and binding characteristics, we hypothesized that they would have similar mechanisms of action. Macrophages were infected with *L. amazonensis* promastigotes for 24 hours, then washed and treated with Fc construct and IFN-γ. The percent-infected macrophages was significantly decreased in the Fc construct and IFN-γ treated cells similar to cells activated with soluble immune complexes, IFN-γ, and *Leishmania* antigen (Figure 4).

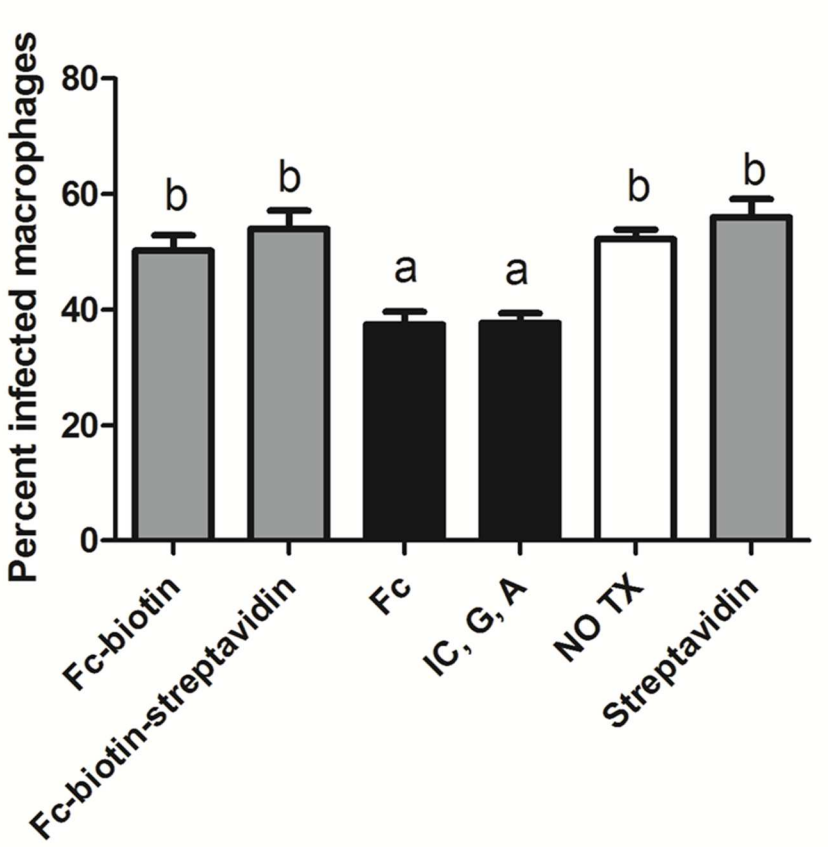

Figure 4. Recombinant IgG2a Fc construct activation of macrophages kills *L. amazonensis* within infected RAW 264.7 macrophages post-infection. Macrophages were infected with *L. amazonensis* promastigotes for 24 hours then cells were washed and treated for 24 hours. Treatments included biotinylated Fc-construct (Fc-biotin) (5 µg/ml) with IFN- γ (5 ng/ml), biotinylated Fc-construct bound to streptavidin (Fc-biotin-streptavidin) (5 µg/ml) with IFN- γ (5 ng/ml), Fc-construct (Fc) (5 µg/ml) with IFN-γ (5 ng/ml), soluble immune complexes (IC) (10 µg/ml) with IFN-γ (5 ng/ml) (G) and *Leishmania* antigen (50 µg/ml (A), no treatment, or streptavidin alone (5 µg/ml). The percent-infected macrophages were determined via light microscopy. Results are pooled data from 4 separate experiments.

Macrophages incubated with Fc construct upregulate superoxide production

Since we know that soluble immune complexes upregulate superoxide, we wanted to demonstrate that the Fc construct would act in a similar manner.[3] We have demonstrated that the constructs are able to produce similar activation of macrophages to reduce the parasite load, we hypothesized that these Fc

constructs are inducing superoxide production in order to kill the intracellular parasites, similar to soluble immune complexes. *L. amazonensis* infected murine macrophages incubated with Fc construct increased superoxide production compared to untreated *L. amazonensis* infected cells (Figure 5).

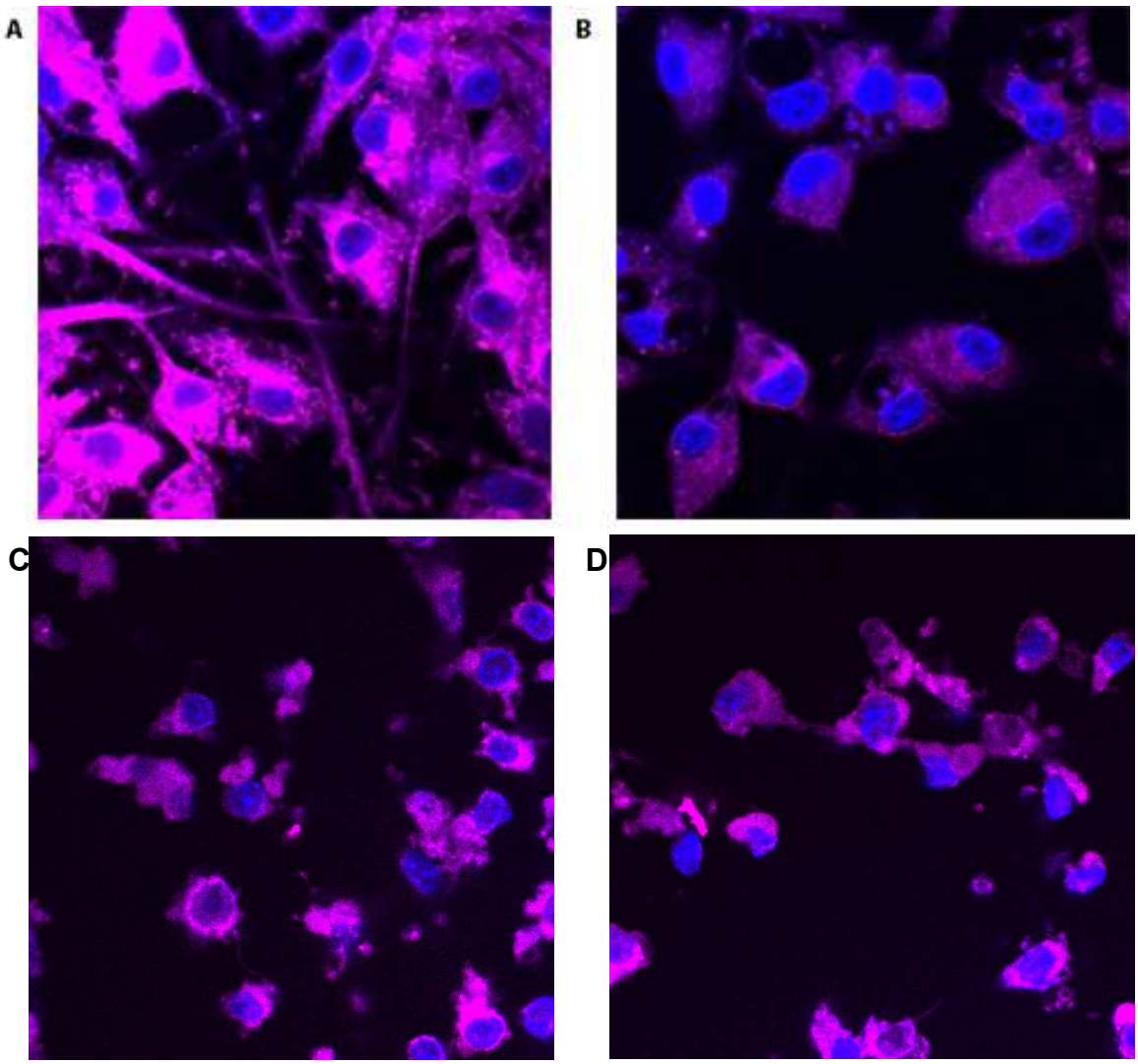

Figure 5. Fc constructs increase superoxide production. A and B: RAW 264.7 macrophages infected with *L. amazonensis* incubated for 24 hours with biotinylated recombinant IgG2a Fc construct bound to streptavidin and IFN-γ (A) or with streptavidin and IFN-γ (B). C and D: Uninfected RAW 264.7 macrophages were incubated for 12 (C) and 24 (D) hours with Fc-construct. Nuclei were stained with Dapi. Superoxide was visualized with confocal microscopy utilizing CellROX (Life Technologies).

Recombinant murine IgG2a Fc construct produced a peak of superoxide production at 2 hours post treatment. Biotinylated Fc construct bound to streptavidin had a peak of superoxide production at 1 hour post treatment and remained elevated compared to other groups at 12 hours post treatment (Figure 6). These differed from the peak of soluble immune complex superoxide production, which occurred at approximately 12 hours post treatment (data not shown).

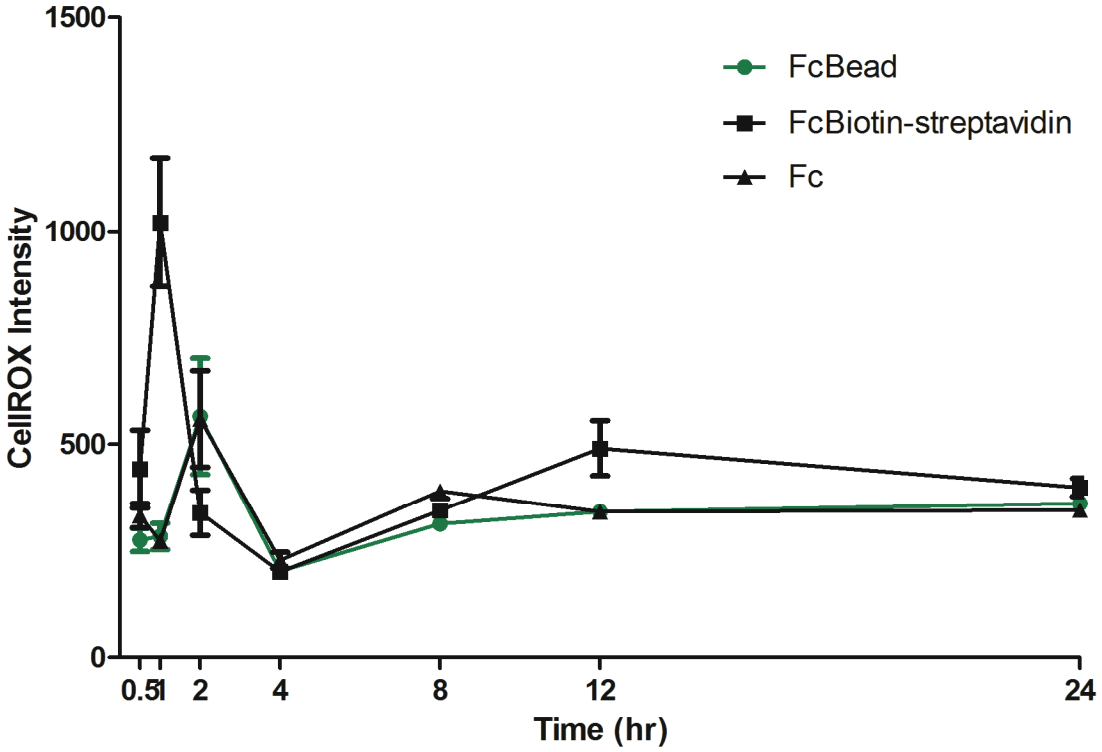

Figure 6. Biotinylated recombinant IgG2a Fc activated superoxide production at approximately 1 hour post activation in RAW 264.7 macrophages with a greater intensity than Fc dimers and Fc coated beads, this remained elevated at 12 hours post treatment. RAW 264.7 cells were activated with biotinylated recombinant IgG2a Fc construct and IFN-γ, Fc-construct and IFN-γ, or Fc-construct coated beads and IFN-γ. Nuclei were stained with Dapi. Superoxide production was measured with confocal microscopy using CellROX (Life

Technologies) and pixel intensity was measured with Image J. The data is pooled from two separate experiments

Inhibition of endocytosis blocks the uptake of soluble immune complexes and Fc construct

Since the non-specific immune complexes required in our system to activate macrophages are small and soluble, we hypothesized that these soluble immune complexes and the Fc construct with similar morphology were taken up via endocytosis. To test this hypothesis we incubated cells with inhibitors of endocytosis or phagocytosis, Dynasore and Cytochalasin D, respectively. Preliminary results revealed that Dynasore blocked the uptake of Fc construct and soluble immune complexes when visualized with confocal microscopy. Cytochalasin D, an inhibitor of phagocytosis, did not block the uptake of Fc construct or soluble immune complexes (data not shown).

Discussion

Here we have described the production of a recombinant murine Fc construct with homologous morphology to murine IgG2a Fc. These constructs can be produced by the transfection of HEK293F cells and protein purification. This allows for larger scale of production of a purified protein product that can be strategically analyzed and manipulated as needed. The ability to develop recombinant proteins can have many potential uses in the manipulation of the immune response. However, more research is needed to understand the molecular mechanisms involved in the activation of these specific intracellular

events. The production of such products could lead to the development of new therapeutic strategies by targeting receptors that have a known therapeutic response or those whose actions are newly being discovered

Intracellular infections can occur due to numerous chronic infectious diseases and their resolution is often complex and requires a robust CD4+ Th1, cell-mediated immune response. This type of immune response is characterized by the production of IFN-γ and often superoxide. Activating an infected cell to undergo a Th1 immune response with the production of IFN-γ and superoxide can lead to clearance of the intracellular infection.

We only saw killing of the parasite with the addition of Fc-constructs rather than with biotinylated Fc or streptavidin bound biotinylated Fc. This may be due to the fact that these constructs will form soluble multimers, which may lead to the clustering of the Fc receptors and the induction of prolonged superoxide production. In contrast biotinylation or streptavidin binding of the biotinylated Fc may lead to large aggregates and subsequent phagocytosis interfering with uptake of these particles by endocytosis and the production of prolonged superoxide production.

In this study we have shown that not only can this recombinant Fc construct be produced, but it also has similar functions in activating the macrophages as compared to murine IgG2a. The Fc construct can bind to murine macrophages *in vitro* and elicit the activation of the macrophages through the production of superoxide and killing intracellular *L. amazonensis*.

References

1. Ashford, R.W. The leishmaniases as emerging and reemerging zoonoses. *Int J Parasitol* **30**, 1269-1281 (2000).

2. Jones, D.E., Ackermann, M.R., Wille, U., Hunter, C.A. & Scott, P. Early enhanced Th1 response after Leishmania amazonensis infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infection and Immunity* **70**, 2151-2158 (2002).

3. Gibson-Corley, K.N. *et al.* An In Vitro Model of Antibody-Enhanced Killing of the Intracellular Parasite Leishmania amazonensis. *PLoS One* **9**, e106426 (2014).

4. Barb, A.W. Intramolecular N-Glycan/Polypeptide Interactions Observed at Multiple N-Glycan Remodeling Steps through [(13)c,N-15]-N-Acetylglucosamine Labeling of Immunoglobulin G1. *Biochemistry-Us* **54**, 313-322 (2015).

5. Raghavan, M. Fc Receptors and their interactions with immunoglobulins. *Cell Dev Biol* (1996).

6. Bonetto, S., Spadola, L., Buchanan, A.G., Jermutus, L. & Lund, J. Identification of cyclic peptides able to mimic the functional epitope of IgG1-Fc for human Fc gamma RI. *Faseb Journal* **23**, 575-585 (2009).

7. Harrison, P.T., Davis, W., Norman, J.C., Hockaday, A.R. & Allen, J.M. Binding of monomeric immunoglobulin G triggers Fc gamma RI-mediated endocytosis. *The Journal of biological chemistry* **269**, 24396-24402 (1994).

8. Pfefferkorn, L.C., Guyre, P.M. & Fanger, M.W. Functional comparison of the inductions of NADPH oxidase activity and Fc gamma RI in IFN gamma-treated U937 cells. *Molecular immunology* **27**, 263-272 (1990).

9. Huang, Z.Y. *et al.* Differential kinase requirements in human and mouse Fc-gamma receptor phagocytosis and endocytosis. *Journal of Leukocyte Biology* **80**, 1553-1562 (2006).

10. Willis, H.E., Browder, B., Feister, A.J., Mohanakumar, T. & Ruddy, S. Monoclonal antibody to human IgG Fc receptors. Cross-linking of receptors induces lysosomal enzyme release and superoxide generation by neutrophils. *Journal of immunology (Baltimore, Md. : 1950)* **140**, 234-239 (1988).

11. Crockett-Torabi, E. & Fantone, J.C. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fc gamma receptor-specific mechanisms. *Journal of immunology (Baltimore, Md. : 1950)* **145**, 3026-3032 (1990).

CHAPTER 5

GENERAL CONCLUSIONS

Summary

The work presented in this dissertation demonstrates the ability to activate macrophages in vitro with soluble immune complexes, IFN-γ, and *Leishmania* antigen in order to kill intracellular *Leishmania amazonensis* in an established infection. This describes a novel mechanism in which antibodies can be used for immune modulation to kill parasites. A major component of the tripartite activation is the production of soluble immune complexes. Our findings show the importance of a B cell response in this model. In our first studies we attempted to knock down B cells for a model of B cell deficient leishmaniasis. However, our findings indicated that despite depletion of B cells with anti-CD20 C3H mice are still able to resolve cutaneous lesions due to a coinfection with *L. major* and *L. amazonensis* and an antibody response persisted (or vice versa).

We were also able to determine important immune factors that were involved during the tripartite activation, which led to the clearance of the parasite via an upregulation of autophagy, seen as LC3 colocalization. We showed that the tripartite activation of macrophages led to a significant increase in superoxide production, which we know is an important factor in killing *L. amazonensis*. This upregulation of superoxide is maintained over 12 hours, which is much longer than other studies have found with superoxide production. We were also able to determine that the activation of superoxide production was related to the upregulation of autophagy through the colocalization of LC3 with parasites within

parasitophorous vacuoles. It has been previously demonstrated that this tripartite activation depended upon activation of the FcRγ (fragment crystallizable) common-chain and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-generated superoxide and here we show a requirement for the protein kinase C (PKC) δ pathway. We hypothesize that the stimulation of the FcRγ and a C-type lectin receptor activate spleen tyrosine kinase (Syk) and proto-oncogene tyrosine protein kinase (Src) pathways, which lead to NADPH oxidase production and the formation of diacylglycerol (DAG) on *Leishmania* containing parasitophorous vacuoles. DAG then recruits PKCδ to the parasitophorous vacuole, which can lead to the activation of autophagy. (Figure 1) There are three different pathways that lead to the formation of DAG. 1) Phospholipase D (PLD) converts phosphatidylcholine (PC) to phosphatidic acid (PA), which is then converted to DAG by phosphatidic acid phosphatase (PAP); 2) Phosphatidylinositol bisphosphate (PIP2) is converted to DAG by phospholipase C (PLC); 3) sphingomyelin synthase (SMS) converts phosphatidylcholine (PC) to DAG. We hypothesize that in our system, DAG is formed through the conversion of phosphatidic acid to DAG by a phosphatidic acid phosphatase enzyme. This would be similar to the antibacterial autophagy pathway shown by Shahnazari et al.[1]

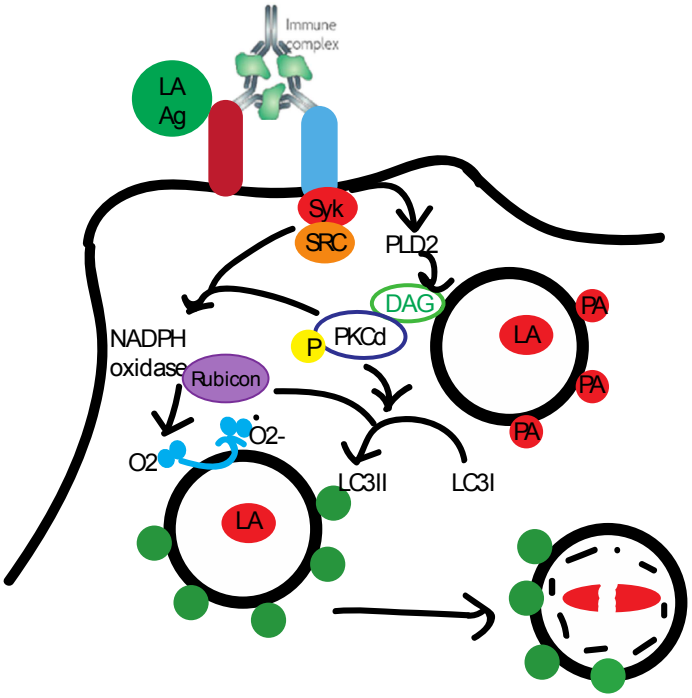

Figure 1. Hypothesized signaling pathway for macrophages infected with *Leishmania amazonensis* (LA) and activated with soluble immune complexes, IFN-γ, and *Leishmania* antigen. This pathway leads to the killing of the intracellular parasite by signaling through NADPH oxidase and non-canonical autophagy pathways, along with a yet unknown c-type lectin receptor (likely Dectin-1). phosphatidic acid (PA), phospholipase D (PLD2) See text for other abbreviations.

Finally, we demonstrated that a recombinant murine IgG2a Fc construct may be able to replace the use of non-specific soluble immune complexes in our system. These Fc constructs can be developed in large quantities of a purified product or other engineering opportunities, such as different glycosylation patterns. These constructs can then be used to manipulate the immune response to promote killing of an intracellular pathogen. Our results demonstrated that these constructs are likely taken up via endocytosis and can induce the macrophages to produce superoxide after being taken up by the FcRγI.

References

1. Shahnazari, S., Namolovan, A., Klionsky, D.J. & Brumell, J.H. A role for diacylglycerol in antibacterial autophagy. *Autophagy* **7**, 331-333 (2011).

Recommendations for future studies

An important goal for future studies is to elucidate the specific signaling pathways that are involved in the tripartite activation of macrophages and the clearance of the intracellular parasites. This would include the important receptors required for activation of the coincidence receptor system. We currently know that the FcRγ common chain and IFN-γ receptor are important in this system but we are uncertain as to the 3rd receptor that is required for the tripartite activation. We hypothesize that a C-type lectin receptor is the 3rd receptor required and determining the specific receptor is an important step in future studies in order to be able to use specific targets or to further the development of a therapeutic. Numerous studies link the Dectin-1 receptor to the production of superoxide and the use of Dectin-1 knockout mice would help to elucidate whether this receptor plays an integral role in the tripartite activation and killing of *Leishmania*.

While we have determined that the autophagy pathway involved is mediated through the PKCδ pathway, it would again be essential to determine which other molecules are involved in this autophagy pathway, specifically determining how DAG is activated. The use of PLC, SMS, or PAP inhibitors would help determine which pathway is leading to the formation of DAG in our

system. Appropriate knock out models and cell lines with transfected GFP-LC3 would also be crucial in determining these pathways.

Other future studies that could be performed would include testing the tripartite activation of macrophages on other intracellular pathogens such as *Salmonella*, *Rhodococcus*, *Brucella*, and *Mycobacterium*, along with many others. The activation of macrophages to induce killing of intracellular pathogens could be a novel way to decrease infections without the use of antibiotics, which essentially could decrease antibiotic use and the development of antibiotic resistance.

Another important goal of this research would be to determine the mechanisms of activation of the Fc constructs and determine why they produce different results than the soluble immune complexes in certain circumstances, such as superoxide production. It would be important to characterize the structure of the Fc constructs and whether or not they are present in dimers or larger constructs. Our findings showed that the superoxide production that was due to Fc constructs occurred much earlier than the soluble immune complexes. The explanation of these differences would require further experiments with purified Fc constructs.

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