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## Regulating Immune Mediated Pathology In Cutaneous Leishmaniasis: Roles For Il-22 And Skin Microbiota

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# Regulating Immune Mediated Pathology In Cutaneous Leishmaniasis: Roles For IL-22 And Skin Microbiota

## Abstract

Cutaneous leishmaniasis is a chronic disease characterized by ulcerating and disfiguring skin lesions. Infection with different species of *Leishmania* parasites is responsible for the initiation of this disease, yet most of the pathology observed is mediated by an unregulated immune response. The work presented in this thesis investigated the roles of IL-22 and the skin microbiota in regulating immune mediated pathology during cutaneous leishmaniasis. We found that IL-22, a cytokine important in wound repair in the skin, was required to limit pathology when mice were infected with *L. major*. In order to promote lesion resolution, IL-22 induced keratinocyte migration and decreased IL-1 $\alpha$  and IL-1 $\beta$  production, both important stages in tissue repair. Interestingly, this protective role for IL-22 was only observed with a high dose of infection, suggesting a threshold of inflammation is required for IL-22 to limit pathology. We also found that the *L. major* infection in mice, as well as, *L. braziliensis* infection in humans caused a dysbiosis in the skin microbiota on lesional skin and nearby skin sites, characterized by a dominance of *Staphylococcus* spp. or *Streptococcus* spp. Interestingly, this dysbiotic microbiota was also transmissible to co-housed naïve skin and exacerbated skin inflammation during *L. major* infection and during an acute contact hypersensitivity model. These data are the first to demonstrate that a dysbiotic skin microbiota can be transmitted to non-inflamed tissue and demonstrate how a naturally occurring dysbiosis can worsen disease during cutaneous leishmaniasis. Work presented in this thesis demonstrates that both IL-22 and the skin microbiota have distinct roles during cutaneous leishmaniasis. Future studies will be aimed at how these factors can be regulated to aid in the treatment of the disease.

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REGULATING IMMUNE MEDIATED PATHOLOGY IN CUTANEOUS LEISHMANIASIS: ROLES  
FOR IL-22 AND SKIN MICROBIOTA

Ciara Gimblet

A DISSERTATION

in

Cell and Molecular Biology

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in

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2017

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FOR IL-22 AND SKIN MICROBIOTA

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## DEDICATION

I especially dedicate this thesis to my nieces and nephews. Ever since the day they were born, I have wanted to be someone they could look to for support and guidance. I sincerely hope that I have made them proud. This work is also dedicated to my entire village, including my husband, parents, siblings, grandparents, aunts, uncles, cousins, friends that are practically family, lab members that are practically family, and of course my two adorable cat children. All of the love and emotional support that you all have given me can never be repaid. Your dedication to my success and happiness is the reason this thesis exists today. Thank you so much and I love you all truly and deeply.

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belief in me has sustained me throughout all of my obstacles. I can never thank them enough.

## ABSTRACT

### REGULATING IMMUNE MEDIATED PATHOLOGY IN CUTANEOUS LEISHMANIASIS: ROLES FOR IL-22 AND SKIN MICROBIOTA

Ciara Gimblet

Phillip Scott, Ph.D.

Cutaneous leishmaniasis is a chronic disease characterized by ulcerating and disfiguring skin lesions. Infection with different species of *Leishmania* parasites is responsible for the initiation of this disease, yet most of the pathology observed is mediated by an unregulated immune response. The work presented in this thesis investigated the roles of IL-22 and the skin microbiota in regulating immune mediated pathology during cutaneous leishmaniasis. We found that IL-22, a cytokine important in wound repair in the skin, was required to limit pathology when mice were infected with *L. major*. In order to promote lesion resolution, IL-22 induced keratinocyte migration and decreased IL-1 $\alpha$  and IL-1 $\beta$  production, both important stages in tissue repair. Interestingly, this protective role for IL-22 was only observed with a high dose of infection, suggesting a threshold of inflammation is required for IL-22 to limit pathology. We also found that the *L. major* infection in mice, as well as, *L. braziliensis* infection in humans caused a dysbiosis in the skin microbiota on lesional skin and nearby skin sites, characterized by a dominance of *Staphylococcus* spp. or *Streptococcus* spp. Interestingly, this dysbiotic microbiota was also transmissible to co-housed naïve skin and exacerbated skin inflammation during *L. major* infection and during an



acute contact hypersensitivity model. These data are the first to demonstrate that a dysbiotic skin microbiota can be transmitted to non-inflamed tissue and demonstrate how a naturally occurring dysbiosis can worsen disease during cutaneous leishmaniasis. Work presented in this thesis demonstrates that both IL-22 and the skin microbiota have distinct roles during cutaneous leishmaniasis. Future studies will be aimed at how these factors can be regulated to aid in the treatment of the disease.

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## CHAPTER 1: INTRODUCTION

### 1.1 Leishmaniasis

Leishmaniases are a group of diseases caused by infection with protozoan parasites belonging to the genus, *Leishmania* spp. Affecting people in over 90 countries worldwide, leishmaniasis has had a major impact on human health. Yet, it still remains a neglected tropical disease (CDC 2013). There are over 20 species of the parasite that cause disease in humans and about 30 species of phlebotomine sand flies that carry the parasite. In the sand fly, leishmania parasites mature into metacyclic promastigotes, which are injected into the skin of the mammalian hosts during blood feeding. Once injected into the skin, the promastigotes are taken up by phagocytic cells, where they transform into the replicative amastigote stage. After multiple rounds of replication, these amastigotes burst from the cell and then can infect other phagocytic cells. The infected phagocytes are then taken up upon blood feeding by another sand fly, where the parasites differentiate into the promastigote stage, completing the life cycle (Kaye and Scott 2011).

Depending on the species, the disease can manifest in several forms: visceral, cutaneous, diffuse cutaneous and mucocutaneous. Visceral leishmaniasis, caused mostly by *Leishmania donovani* and *Leishmania infantum*, affects several internal organs causing fever, weight loss and anemia and untreated cases of visceral leishmaniasis are almost always fatal. Cutaneous

leishmaniasis is the most common form throughout the world and can manifest in ulcerating lesions, many of which can resolve but leave disfiguring scars. The predominant species that cause cutaneous leishmaniasis are the Old World species, *Leishmania major*, and the New World species *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania panamanensis*. Some individuals infected with *L. braziliensis* and *L. panamanensis* develop chronic ulcers that have the potential to progress to the mucocutaneous form, which can cause severe disfiguration (Kedzierski 2011).

*Leishmania* parasites can infect a variety of mammalian hosts, including humans, dogs, and rodents. In certain countries where leishmaniasis is endemic, infected domestic dogs are considered the predominant reservoir for *leishmania* parasites and have been targeted to help prevent the spread of the disease (Oliveira et al. 2008; Lima et al. 2012; Esch et al. 2012). Control of the sand fly vector has also been tested as a measure to help prevent new infections, but it is not yet clear whether it will be useful for all forms of the disease (Gonzalez et al. 2015). The development of an effective vaccine would provide an additional measure of disease control and is an area of active investigation.

Pentavalent antimonial therapies have been the first line of treatment for patients with leishmaniasis for almost 70 years. However many patients require multiple rounds of treatment before a successful cure is reached, and can experience severe side effects due to drug toxicity (Kedzierski 2011). Currently, there is no protective vaccine against human leishmaniasis despite many

experimental efforts and large human trials over the past 25 years (Basu et al. 2005; Chakravarty et al. 2011; Bhattacharya et al. 2015; Miura et al. 2015) Most vaccine strategies have been focused on developing strong antibody responses, yet prior studies have shown that antibodies have no protective effect on the disease (Glennie and Scott 2016). The success of future vaccine strategies depends on a thorough understanding of how protective immunity is achieved.

## **1.2 Protective immune responses in leishmaniasis**

Leishmania parasites also infect and cause disease in mice, making the murine model of infection useful in studying the immune response. For cutaneous leishmaniasis, *L. major* has been the most widely used parasite in these studies. Seminal work using the mouse model demonstrated that susceptibility to infection with leishmania parasites depends on the strain of mouse (Kellina 1973). Subsequent studies focused on understanding the immunological differences that lead to susceptibility and resistance to leishmania. Several studies demonstrated that an IL-4 driven Th2 immune response was responsible for uncontrolled parasite growth in susceptible mouse strains, while the production of IFN- $\gamma$  from activated Th1 cells were responsible for control of parasite growth (Scott et al. 1988; Heinzel et al. 1989; Sadick et al. 1990; Heinzel et al. 1993; Sypek et al. 1993; Kopf et al. 1996). IL-12 is the key cytokine that initiates the protective Th1 response (Mattner et al. 1996; Scharon-Kersten et al. 1995). Upon infection with leishmania parasites, reactive oxygen species (ROS)



are induced in phagocytic cells. This response, known as the respiratory burst can kill a portion of the parasites. However, a stronger anti-parasitic response requires the release of IFN- $\gamma$ , which induces infected cells to produce more ROS and nitric oxide (NO) (Scott and Novais 2016). Interestingly, unlike in murine macrophages, NO is not induced or required for parasite killing in human cells which rely more on the respiratory burst (Novais et al. 2014b). This data suggests that mice and humans eliminate the parasite using different mechanisms. Nonetheless, it is clear that in both mice and humans, IFN- $\gamma$  is required to effectively control parasite growth.

There are several sources of IFN- $\gamma$  during leishmania infections, including CD4 T cells, CD8 T cells,  $\gamma\delta$  T cells, and NK cells. While CD4 T cells are the most crucial source for parasite control (Chakkalath et al. 1995; Erb et al. 1996), IFN- $\gamma$  from CD8 T cells and NK cells is important in fortifying the protective Th1 response (Belkaid et al. 2002b; Scharon and Scott 1993; Uzonna, Joyce, Scott 2004; Laouar et al. 2005). In addition, TNF- $\alpha$  acts synergistically with IFN- $\gamma$  to enhance parasite killing (Bogdan et al. 1990; Green et al. 1990). These studies demonstrate the need for multiple arms of the immune response working together to effectively control parasite growth.

The innate response also plays a role in contributing to the protective Th1 immune response. Upon recognition of the parasite, NK cells are responsible for the early IFN- $\gamma$  production (Scharon and Scott 1993; Bajenoff et al. 2006). Even cells from the non-hematopoietic niche contribute to initiating and maintaining a

protective Th1 response. Keratinocytes can be an early source of IL-12, as well as other cytokines that promote IFN- $\gamma$  production (Ehrchen et al. 2010).

Even in the face of a strong Th1 response, leishmania parasites persist after the resolution of the lesion, leading to a concomitant immunity that protects against reinfection. The mechanism of how the parasite persists is not completely clear, but the production of the regulatory cytokine, IL-10, plays a role by dampening the Th1 response (Belkaid et al. 2001; Belkaid et al. 2002a). It is thought that protection against reinfection requires parasite persistence and that this immunity is dependent upon both CD4 and CD8 memory T cells (Muller 1992). In cutaneous leishmaniasis, both short-lived effector memory T cells and long-lived central memory T cells are required for protection against re-infection (Peters et al. 2014; Zaph et al. 2004). More recently, skin resident CD4 T cells have also been identified in *L. major* immune mice. These cells remain in the skin long after the primary infection where they produce IFN- $\gamma$  in response to the parasite and recruit circulating T cells in the skin to help further prevent parasite growth (Glennie et al. 2015). The immunity provided by these multiple subsets of memory T cells makes them ideal vaccine targets. Additional studies are necessary to learn about how these cells are maintained.

While we understand the type of immune response generated during leishmania infection and how parasite replication is controlled, we still do not fully comprehend how long-lasting immunity is acquired or how to effectively translate that immunity to a protective vaccine in humans. The treatment for cutaneous

leishmaniasis also needs to be improved and may require regulation of the immune response to achieve effective therapy. In the next section, we discuss the role of the immune response in the pathogenesis of leishmania infections.

### **1.3 Immune mediated pathology in cutaneous leishmaniasis**

Pathology in cutaneous leishmaniasis is not always a consequence of uncontrolled parasite growth. In fact, even though patients infected with leishmania show signs of overt pathology, there are sometimes very few parasites found in the lesions (Nylen and Eidsmo 2012). Instead, much of the disease observed in cutaneous leishmaniasis has been associated with immune mediated pathology. Interestingly, the same immune responses that provide protection exacerbate the disease when the responses are not controlled. TNF- $\alpha$  and IFN- $\gamma$  both help promote the Th1 response necessary to kill leishmania parasites. Yet, patients with severe, chronic cutaneous and mucocutaneous leishmaniasis express higher levels of these cytokines (Bacellar et al. 2002; Gaze et al. 2006; Melby et al. 1994). These studies suggest that the balance between parasite control and tissue protection must be carefully regulated in order to limit disease. In fact, patients treated with drugs targeting the TNF- $\alpha$  pathway in combination with anti-leishmania drugs, experience faster healing times and higher cure rates than patients with anti-leishmania treatment alone (Lessa et al. 2001; Bafica et al. 2003; Machado et al. 2007; Ribeiro de Jesus et

al. 2008). In the treatment of cutaneous leishmaniasis, it is thus necessary to consider the effects of immunotherapy in addition to anti-parasitic drugs.

Other cytokines have been implicated in mediating pathology during cutaneous leishmaniasis as well. IL-1 $\beta$  and IL-1 $\alpha$  are cytokines produced mainly by innate cells as well as non-hematopoietic cells, which in the skin are mostly keratinocytes. While they have important anti-microbial properties, these cytokines can also lead to skin inflammation in diseases including psoriasis, atopic dermatitis, and cutaneous lupus erythematosus (Mee et al. 2006; Rauschmayr, Groves, Kupper 1997; Shepherd, Little, Nicklin 2004; Mattii et al. 2013; Nutan, Kanwar, Parsad 2012; Jensen 2010). IL-1 family cytokines, in particular IL-1 $\beta$ , has also been implicated in mediating disease during cutaneous leishmaniasis. In humans, IL-1 $\beta$  expression correlates with more severe cutaneous leishmaniasis (Fernandez-Figueroa et al. 2012; Novais et al. 2014a). These data suggest that IL-1 $\beta$  plays a role in mediating pathology in the skin. Yet, how this immunopathology occurs remains unclear. IL-1 $\beta$  can lead to the production of chemokines, which can recruit inflammatory cells into the skin, and matrix metalloproteinases, which break down the extracellular matrix and damages the skin. This relationship between IL-1 $\beta$  and neutrophil recruitment to lesional skin has also been demonstrated in mice infected with *L. major* (Voronov et al. 2010; Gonzalez-Lombana et al. 2013; Charmoy et al. 2016). From these studies, it is clear that IL-1 $\beta$  and neutrophils exacerbate pathology during cutaneous leishmaniasis, while having minimal effect on parasite control. IL-1 $\beta$  recruits neutrophils and other inflammatory cells to the skin, but can also drive

the development and maintenance of Th17 cells (Sutton et al. 2006; Yang et al. 2008; Ikeda et al. 2014), which are known to drive inflammation in the skin during cutaneous leishmaniasis in humans (Souza et al. 2012) as well as in mice (Lopez Kostka et al. 2009; Anderson et al. 2009; Gonzalez-Lombana et al. 2013). Using a non-healing mouse model of cutaneous leishmaniasis, our lab has demonstrated that IL-10 signaling is required to limit an IL-17-mediated pathology. Although infection with *L. major* normally resolves in a C57BL/6 mouse, we observed increased ulceration and immunopathology that was not ameliorated unless we neutralized IL-17. It was previously believed that IFN- $\gamma$  and TNF- $\alpha$  were the main drivers of the immunopathology associated with cutaneous leishmaniasis, however our recent studies indicate that Th17 cells also play a critical role and warrant further investigation into other factors that could be important in lesion resolution and pathology (Anderson et al. 2009; Lopez Kostka et al. 2009; Pitta et al. 2009; Gonzalez-Lombana et al. 2013; Banerjee et al. 2016)

The lack of regulatory cytokines has proven to be just as important as the presence of pro-inflammatory cytokines. IL-10, a cytokine known for its ability to limit inflammation has a prominent role during cutaneous leishmaniasis as it down-regulates the Th1 response during infection. This role proves important because it can limit inflammation as well as maintain a low-level of parasites necessary to develop long-lasting immunity (Belkaid et al. 2001; Belkaid et al. 2002a; Anderson et al. 2007). However, low expression of IL-10 and/or the IL-10 receptor has been associated with more severe disease during cutaneous and

mucocutaneous leishmaniasis in human patients (Bacellar et al. 2002; Faria et al. 2005; Gaze et al. 2006; Gomes-Silva et al. 2007). While results from the mouse model can lead to varying results depending on the strain of the mouse, it is evident that IL-10 is critical in modulating the immune response during cutaneous leishmaniasis (Kane and Mosser 2001; Gonzalez-Lombana et al. 2013).

T cells produce these pro-inflammatory and anti-inflammatory cytokines and are some of the main cell types that regulate immune mediated pathology during cutaneous leishmaniasis. CD4<sup>+</sup> T cells are a major source of inflammatory cytokines including IFN- $\gamma$ , TNF- $\alpha$  and IL-17 in the skin. And there is clear evidence that CD4<sup>+</sup> T cells mediate pathology during leishmania infections. RAG deficient mice, which lack T and B cells, are unable to control parasite growth, yet do not develop skin pathology in response to several species of leishmania until quite late in the infection (Terabe et al. 1999; Belkaid et al. 2002b; Novais et al. 2013). However, these mice develop lesions when they receive CD4<sup>+</sup> T cell transfers (Soong et al. 1997) during *L. amazonensis* infection suggesting that CD4<sup>+</sup> T cells can drive pathology. CD8<sup>+</sup> T cells can also be a source of inflammatory cytokines in the skin. In fact, when transferred into RAG deficient mice during *L. major* and *L. braziliensis* infections, CD8<sup>+</sup> T cells lead to increased pathology with larger lesion development (Belkaid et al. 2002b; Novais et al. 2013). However, it appears the cytotoxicity, not cytokine production, from CD8<sup>+</sup> T cells is responsible for the tissue damage. During cutaneous leishmaniasis in humans, cytotoxicity from CD8<sup>+</sup> T cells is associated with ulceration and more severe pathology (Faria et al. 2005; Santos Cda et al. 2013;

Novais et al. 2014a; Cardoso et al. 2015). These data combined with studies using mouse models indicate that while CD8+ T cells can help mediate parasite control, they are also critical in the immune mediated pathogenesis during cutaneous leishmaniasis.

During cutaneous leishmaniasis, pro-inflammatory cytokines and the cells that produce them drive the development of lesions and ulceration in the skin. This immune mediated pathology is also present in other skin diseases including psoriasis and atopic dermatitis, in which Th1, Th2, Th17, and cytotoxic CD8+ T cells all have distinct roles in causing a breakdown in the barrier integrity of the skin (Di Cesare, Di Meglio, Nestle 2009; Pantelyushin et al. 2012; Martin et al. 2012; Guilloteau et al. 2010; Hijnen et al. 2013; Di Meglio et al. 2016; Hennino et al. 2007; Hennino et al. 2011). Therapies designed to dampen the inflammatory response are currently being used in psoriasis and atopic dermatitis, but not to a large degree in cutaneous leishmaniasis. In cutaneous leishmaniasis, immunotherapies remain a complicated issue due to the fact that the development of a protective immune response is necessary to control the parasite. In this thesis, we will define some of the factors that influence the immune responses mediating damage to the skin during cutaneous leishmaniasis.

#### **1.4 The role of Interleukin-22 in skin inflammation**

There are many regulators, positive and negative, of inflammation in the skin, but one of particular interest is IL-22 due to its role in wound healing and inflammation in the skin. In the past few years there has been increased focus on the IL-10 family of cytokines, which consist of IL-10, IL-19, IL-20, IL-26, the  $\lambda$ -interferons, and probably its most studied member, IL-22. IL-22 is expressed in several tissues throughout the body including, the thymus, brain, liver, gut, lung, pancreas, spleen, and skin, making its potential effects widespread (Dumoutier, Louahed, Renauld 2000; Wolk et al. 2004). IL-22 is produced by several cellular sources including, CD4+ T cells, CD8+ T cells,  $\gamma\delta$  T cells, NKT and NK cells, and several innate lymphoid cell populations (Wolk et al. 2002; Martin et al. 2009; Spits et al. 2013; Cella et al. 2009; Goto et al. 2009). Non-lymphoid sources, including macrophages, neutrophils, and fibroblasts, have been reported, but may represent a smaller fraction of production (Hansson et al. 2013; Zindl et al. 2013; Ikeuchi et al. 2005).

IL-22 signals through the heterodimer comprised of IL-22R1, a type two cytokine receptor member of the IL-10 family, and the IL-10R2. Interestingly, IL-22 is different from most other interleukins in that it does not act on immune cells. IL-22R1 expression is limited to non-hematopoietic epithelial cells and fibroblasts. In the skin keratinocytes and fibroblasts are the main targets of IL-22 (Wolk et al. 2004; Brembilla et al. 2016). The effects of IL-22 on target cells are mediated through activation of the Jak1/Tyk2 kinases leading to the phosphorylation of STAT3, primarily, but also STAT1 and STAT5 (Lejeune et al. 2002; Wolk et al. 2004). The activation of these pathways leads to proliferation,



migration, and upregulation of pro-survival genes, while it inhibits differentiation in keratinocytes (Sabat, Ouyang, Wolk 2014). These effects of IL-22 are important in wound healing, as well as skin inflammation.

As epithelial cells and fibroblasts are the main targets of IL-22, the effects of this cytokine at barrier surfaces are necessary during tissue repair. During intestinal damage induced by colitis, IL-22 causes epithelial cells to proliferate and migrate, ultimately leading to tissue repair and which can protect against tumor formation (Zenewicz et al. 2008; Pickert et al. 2009; Huber et al. 2012). During HIV and SIV infections, IL-22 limits epithelial damage in the intestine and protects against bacterial translocation (Klatt et al. 2012; Kim et al. 2012). Similarly, IL-22 contributes to protection and regeneration of lung epithelial cells during influenza infection (Kumar et al. 2013; Paget et al. 2012; Pociask et al. 2013). This protection also prevented secondary bacterial infections (Ivanov et al. 2013). These effects of IL-22 are also seen in the skin. Keratinocytes are the main target of IL-22 in the skin. Using an in vitro injury model, one study demonstrated that IL-22 induced proliferation and migration of keratinocytes to induce wound closure after damage (Boniface et al. 2005). IL-22 also limits the differentiation of keratinocytes (Boniface et al. 2005; Wolk et al. 2006; Wolk et al. 2009). While continually blocking differentiation could inhibit wound healing, it is helpful in the initial stages when keratinocytes need to regenerate the basal layer of the epidermis. Fibroblasts can also respond to IL-22 and contribute to wound healing after injury. IL-22 induces myofibroblast differentiation and helps with wound closure in an acute injury model (McGee et al. 2013). Wound healing is

an important feature during cutaneous leishmaniasis involving a regulated response of keratinocyte survival, fibroblast maturation, and collagen deposition (Eidsmo et al. 2005; Eidsmo et al. 2007; Tasew et al. 2010; Almeida et al. 2015; Sakthianandeswaren et al. 2005; Baldwin et al. 2007; Elso et al. 2004b; Elso et al. 2004a), but whether IL-22 has these effects during infection are not yet known.

The effects of IL-22 on wound healing can also lead to inflammation and pathology depending on the context. In addition to inducing proliferation and migration, IL-22 also induces chemokine expression in epithelial cells. Neutrophilic-attracting chemokines like CXCL1, CXCL2, CXCL5, and CXCL8 are induced in epithelial cells after exposure to IL-22 (Aujla et al. 2008; Wolk et al. 2009). These chemokines create a cascade of inflammation that can cause more damage than repair. These effects are amplified in combination with other inflammatory cytokines including IL-17, TNF- $\alpha$ , and IFN- $\gamma$  (Wolk et al. 2004; Wolk et al. 2009; Guilloteau et al. 2010). In fact, IL-22 limits lung injury in the absence of IL-17, while driving inflammation when IL-17 is present (Sonnenberg et al. 2010). The balance between protection and pathology must also be regulated during cutaneous leishmaniasis, and IL-22 may play a role in that process.

IL-22 also causes inflammation and pathology in the skin. Mice that constitutively express IL-22 or have IL-22 injected into their skin develop severe inflammation in the skin, similar to what is observed during psoriasis and atopic dermatitis (Zheng et al. 2007; Wolk et al. 2009; Ma et al. 2008; Van Belle et al.

2012; Wang et al. 2013). Similar to what is observed during tissue repair, IL-22 induces keratinocyte proliferation during inflammatory skin disorders. However in this scenario, hyperproliferation leads to acanthosis and inflammation that worsens the pathology.

IL-22 also has antimicrobial effects at barrier surfaces. In the intestine, lung, and skin, IL-22 induces the expression of antimicrobial peptides in the  $\beta$ -defensins, S100, and Reg families (Wolk et al. 2004; Zheng et al. 2008; Wolk et al. 2006; Aujla et al. 2008; Brand et al. 2006; Sekikawa et al. 2010). These antibacterial actions lead to protection from invading pathogens, but also regulate the commensal populations in the intestinal tract (Sonnenberg et al. 2012; Zenewicz et al. 2013). However, the modulation of the commensal population does not always lead to protection. IL-22 induces the expression of antimicrobial peptides that suppress *Enterobacteriaceae* colonization by nutrient sequestration in the intestine. However, in the absence of this commensal, the pathogen *Salmonella enterica* is able to better colonize and cause inflammation (Behnsen et al. 2014). During infection with the parasite *Toxoplasma gondii*, IL-22 also drives inflammation in the intestinal tract (Munoz et al. 2009; Wilson et al. 2010), but whether it is due to an imbalance in the commensal bacteria is unknown. Commensal bacteria on the skin drive an inflammatory response that could lead to lesion development in cutaneous leishmaniasis (Naik et al. 2012), but whether the anti-microbial effects of IL-22 can regulate this process is unknown.

IL-22 is an active member of the IL-10 cytokine family at barrier surfaces. The roles of IL-22 in the skin, gut, lung, and liver have demonstrated that this cytokine can affect the outcomes of injury, infection, and inflammation in a variety of ways. During cutaneous leishmaniasis, the processes of wound healing and inflammation must be tightly regulated in order to successfully resolve lesions and control parasite replication. The actions of IL-22 on the skin immunity may be a key player in these processes.

### **1.5 The microbiota and skin immunity**

The microbiota is the collective populations of bacteria, viruses, fungi, protozoa and archaea found in our environment or associated with various tissues and organs throughout our body. It has been estimated that there are from 3-10 times more bacterial cells in the body than human cells (Woese 1987; Sender, Fuchs, Milo 2016), and it is evident that the microorganisms associated with our body are important players in our biology. Bacteria are found in or on many parts of the body, including the intestinal tract, skin, mouth, and the reproductive tract. While the exact numbers may vary depending on size and gender of the person, early studies suggested that the intestinal tract harbored the most bacteria with about  $10^{14}$  cells, followed by the skin with about  $10^{12}$  cells, while the rest of the body sites harbor around  $10^{12}$  bacteria combined (Savage 1977; Berg 1996). Many studies have focused on the bacteria in the intestinal

tract, but recently studying the commensal bacteria on the skin has become a larger area of interest.

Prior to the age of genomics, culture based methods were used to study the bacteria in the environment. However, it became apparent that simply culturing samples was not capturing all the bacteria present (Amann, Ludwig, Schleifer 1995; Staley and Konopka 1985). The discovery that bacterial phylogeny could be determined based on the well-conserved 16S ribosomal RNA (rRNA) gene (Woese 1987) set the stage for the present-day microbiota studies. Presently, bacterial communities are identified using high-throughput sequencing. The Human Microbiome Project was started in 2007 and collected over 200 samples across various body sites in order to define the microbiota of healthy adults. This study used 16S rRNA gene and whole genome sequencing to demonstrate that the different body sites harbored distinct, yet diverse bacterial communities (NIH HMP Working Group et al. 2009). This study, along with many others that followed, suggested that a healthy microbiota is typically a diverse one.

Subsequent studies have shown that perturbations in the microbiota, often referred to as dysbiosis, are associated with disease and inflammation. This association with disease and dysbiosis has been observed during inflammatory bowel disorders, metabolic disorders, cancer, as well as inflammatory skin diseases (Sartor 2009; Garrett et al. 2010; Ley et al. 2005; Turnbaugh et al. 2006; Castellarin et al. 2012; Gao et al. 2008; Kong et al. 2012). While many of

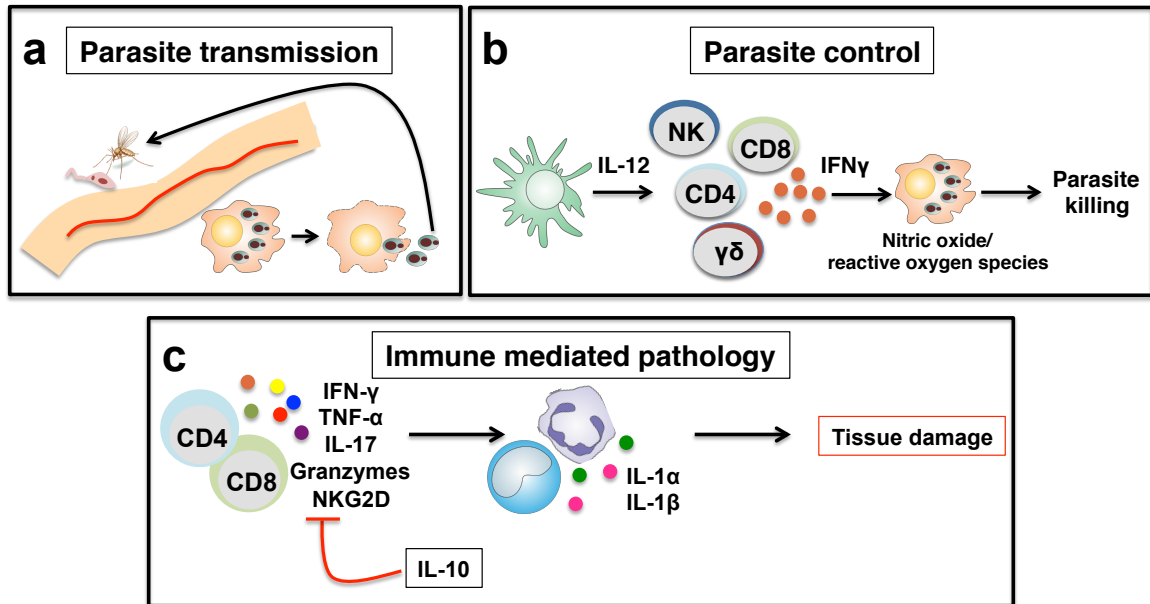
these studies show only correlations between dysbiosis and disease, more recent research has focused on determining whether dysbiosis is a cause or consequence of disease. A lot of those studies have focused on the intestinal tract. Bacteria in the intestinal tract that cause dysbiosis have been shown to drive disease in arthritis, obesity, cancer, and colitis (Wu et al. 2010; Turnbaugh et al. 2006; Wu et al. 2009; Sellon et al. 1998; Elinav et al. 2011a; Zenewicz et al. 2013). These effects are often mediated through modulation of immune responses. Interestingly, there is also evidence that dysbiosis can drive an immune regulatory phenotype and protect against disease in the intestine (Atarashi et al. 2013). It is evident that the intestinal microbiota interacts with the immune system to either drive disease, or protect the host from inflammation. It is not yet evident that the microbiota on the skin are also as important in diseases.

While it is clear that the bacteria on the skin are quite numerous, less is known about how those microorganisms influence immunity in the skin. The diversity of the skin microbiota depends on the body site as well as what type of environment is present at that site. For example, oily, dry, and moist body sites all harbored distinct bacterial communities (Grice et al. 2009). This difference in the types of microbes on different body sites was also observed in fungal and viral communities (Findley et al. 2013; Hannigan et al. 2015). While these studies demonstrated that healthy skin sites typically have diverse microbial populations, the same is not always true during disease in the skin.

Inflammatory skin disorders, including atopic dermatitis, psoriasis, and chronic diabetic wounds, have been associated with changes in the skin microbiota (Kong et al. 2012; Alekseyenko et al. 2013; Gao et al. 2008; Price et al. 2009; Grice et al. 2010; Loesche et al. 2016). However, it is not clear what causes these changes. During inflammation, cytokines, chemokines, and antimicrobial peptides are often produced, potentially explaining why there are changes in the microbiota. Some bacteria, like *Salmonella typhimurium* and *Escherichia coli*, can utilize products of the immune response by changing their metabolic processes. This adaptation allows them to thrive in the face of inflammation, ultimately leading to dysbiosis in the microbiota (Winter et al. 2010; Behnsen et al. 2014; Hughes et al. 2017). This phenomenon is apparent in the intestine (Atherton and Blaser 2009; Behnsen et al. 2014), but it remains unclear whether it occurs in the skin.

But what is clear is that the skin microbiota can influence the cutaneous immune response. Recent studies have shown that prominent members of the skin microbiota, for example, *Staphylococcus* spp. can drive inflammatory Th1 and Th17 responses in the skin. In some cases the cytokines from these cells can lead to protection from a pathogen (Naik et al. 2015), while driving inflammation in response to other pathogens, including *L. major* infection (Naik et al. 2012). Bacteria can even drive the development of regulatory responses in the early stages of life that help limit inflammation during infection experienced as an adult (Scharschmidt et al. 2015). These studies demonstrate how colonization with bacteria can influence skin immunity, but it is unknown whether naturally

occurring dysbiosis has any influence on skin disease. There is some evidence of dysbiosis driving disease in atopic dermatitis (Kobayashi et al. 2015), but whether dysbiosis occurs during leishmania infection or influences disease in cutaneous leishmaniasis remains unknown.



**Figure 1. Leishmania transmission, control, and role in immune mediated pathology. (a)** Sandflies transfer leishmania parasites into the skin upon blood feeding. The parasite is taken up by phagocytic cells, where it can replicate and spread to other cells. The parasite is transmitted back into the sandfly upon another blood feeding. **(b)** IL-12 production from dendritic cell induces IFN- $\gamma$  production from T cells and NK cells. IFN- $\gamma$  activates infected cells to kill the parasite. **(c)** During infection, inflammatory cytokines are produced by T cells. These cytokines recruit inflammatory monocytes and neutrophils, which make IL-1 $\alpha$  and IL-1 $\beta$ . The recruitment of these inflammatory cells ultimately lead to tissue damage. IL-10 can limit this pathology by blocking cytokine production from T cells.

## 1.6 Summary

It is clear that the magnitude of the disease associated with leishmania infections is not only mediated by parasite replication, but also includes the



immune response as a major cause of pathology. Several cytokines have been identified to regulate inflammation during cutaneous leishmaniasis. Inflammatory cytokines, IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , and IL-17 all seem to mediate tissue damage in the course of infection with leishmania parasites. On the other hand, regulatory cytokines like IL-10 are also important to limit the actions of the inflammatory cytokines.

Limiting inflammation and establishing a wound healing response is required to resolve cutaneous leishmaniasis lesions (Baldwin et al. 2007; Elso et al. 2004a; Elso et al. 2004b). IL-22 is a cytokine of interest because of its dual roles in inflammation and wound healing. While there is some evidence that IL-22 is associated with protection during visceral leishmaniasis (Pitta et al. 2009; Ghosh et al. 2013), the role of IL-22 is less well studied during cutaneous leishmaniasis. Here, we will investigate the role of IL-22 during cutaneous leishmaniasis to determine if it is involved in driving inflammation or limiting disease. IL-22 can also regulate the commensal microbiota, another potential way it can affect the immune response in the skin. While there are varying results describing the effects of the microbiota during cutaneous leishmaniasis (de Oliveira et al. 1999; Oliveira et al. 2005; Naik et al. 2012), it is clear that the skin microbiota have a role during this disease. Here, we will investigate whether the microbiota is influenced by cutaneous leishmaniasis and if those naturally occurring changes influence the outcome of disease. The goals of these studies are designed to better understand what factors mediate immunopathology during

cutaneous leishmaniasis, with the hope to develop more effective therapies for the disease.

## CHAPTER 2: IL-22 PROTECTS AGAINST TISSUE DAMAGE DURING CUTANEOUS LEISHMANIASIS

### 2.1 Abstract

Cutaneous leishmaniasis is a disease characterized by ulcerating skin lesions, the resolution of which requires an effective, but regulated, immune response that limits parasite growth without causing permanent tissue damage. While mechanisms that control the parasites have been well studied, the factors regulating immunopathologic responses are less well understood. IL-22, a member of the IL-10 family of cytokines, can contribute to wound healing, but in other instances promotes pathology. Here we investigated the role of IL-22 during leishmania infection, and found that IL-22 limits leishmania-induced pathology when a certain threshold of damage is induced by a high dose of parasites. *Il22<sup>-/-</sup>* mice developed more severe disease than wild-type mice, with significantly more pathology at the site of infection, and in some cases permanent loss of tissue. The increased inflammation was not due to an increased parasite burden, but rather was associated with the loss of a wound healing phenotype in keratinocytes. Taken together, these studies demonstrate that during cutaneous leishmaniasis, IL-22 can play a previously unappreciated role in controlling leishmania-induced immunopathology.

## 2.2 Introduction

Cutaneous leishmaniasis is a major neglected tropical disease affecting about 12 million people globally (Kedzierski 2010). The spectrum of clinical manifestations in cutaneous leishmaniasis ranges from self-limiting nodules to non-healing ulcers with a highly inflammatory immune response, and the disease is caused by several different species of leishmania that reside within phagocytic cells. Control of the parasites requires IFN- $\gamma$  produced by CD4+ Th1 cells (Wang et al. 1994). However in spite of a Th1 response, some patients exhibit severe non-healing lesions (Bacellar et al. 2002; Gaze et al. 2006). Thus, in addition to controlling the parasites, regulating the inflammatory response is essential for disease control. TNF- $\alpha$  (Antonelli et al. 2005; Bafica et al. 2003), IL-1 $\beta$  (Fernandez-Figueroa et al. 2012; Voronov et al. 2010) and IL-17 (Gonzalez-Lombana et al. 2013; Lopez Kostka et al. 2009) have all been implicated in promoting pathology in leishmaniasis, and damage caused by cytolytic CD8 T cells can also contribute to these immunopathologic responses (Crosby et al. 2014; da Silva Santos et al. 2014; Novais et al. 2013; Novais et al. 2014a). IL-10 can regulate some of these immunopathologic responses (Faria et al. 2005; Gonzalez-Lombana et al. 2013). Since drug treatment is often ineffective (Bafica et al. 2003), and no human vaccine exists for the disease, a better understanding of the factors that mediate lesion resolution is essential to help develop new immunotherapies for the disease.

Recently, members of the IL-10 subfamily have been identified as key players in the wound healing process (Sa et al. 2007; Sun et al. 2013; Wolk et al. 2002). IL-22 is a prominent member of this family, and can instruct non-immune cells, such as epithelial cells and fibroblasts, to proliferate, migrate, and mend the extracellular matrix after injury (Boniface et al. 2005; McGee et al. 2013). These functions are important in maintaining surface barrier integrity and protection against subsequent infections. Additionally, IL-22 has been shown to induce the production of antimicrobial peptides from epithelial cells in order to maintain a balanced commensal population and prevent dysbiosis (Sonnenberg et al. 2011; Zenewicz et al. 2013; Zheng et al. 2008). However, while IL-22 is important for tissue protection and contributes to wound healing in the skin, gut, and lungs (Aujla et al. 2008; McGee et al. 2013; Pickert et al. 2009), it can also be pathogenic in other inflammatory conditions, such as psoriasis (Van Belle et al. 2012). These pathologic responses are mediated by some of the same functions of IL-22 that are protective, including uncontrolled proliferation and the production of inflammatory molecules (Ma et al. 2008; Sonnenberg et al. 2010; Van Belle et al. 2012; Zheng et al. 2007). Why IL-22 is protective in some situations and pathogenic in others is unclear, but may depend on the amount of IL-22 produced, as well as the presence of other inflammatory cytokines such as IL-17 (Guilloteau et al. 2010; Sonnenberg et al. 2010).

Like in some patients, the lesions of C57BL/6 mice normally heal after *L. major* infection. In order to determine if IL-22 contributes to resolution of a leishmanial infection, we infected *IL22<sup>-/-</sup>* mice with *Leishmania major* and *L.*

*braziliensis* and monitored the course of infection. We found that *IL22<sup>-/-</sup>* mice exhibited increased tissue pathology compared with infections in wild-type mice. The absence of IL-22 did not influence the parasite burden, but rather led to higher levels of keratin 6a and keratin 16, both of which have been implicated in inhibiting the wound healing capabilities of keratinocytes (Rotty and Coulombe 2012; Wawersik et al. 2001). We discovered that a role for IL-22 was only evident with high doses of parasites, suggesting that a threshold of inflammation might have to be reached before IL-22 contributed to tissue protection. Taken together, our results demonstrate a previously unknown role for IL-22 in limiting pathology during leishmania infection.

## **2.3 Materials and methods**

### **Ethics statement**

This study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines (Ethical Committee of the Maternidade Climerio de Oliveira, Salvador, Bahia, Brazil; and the University of Pennsylvania Institutional Review Board). This study was approved by the Ethical Committee of the Federal University of Bahia (Salvador, Bahia, Brazil) (010/10) and the University of Pennsylvania IRB (Philadelphia, PA) (813390). All patients provided written informed consent for the collection of samples and

subsequent analysis. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01.

### **Mice**

Female C57BL/6 mice 6-8 weeks old were purchased from the National Cancer Institute (Frederick, MD). B6.IL22 (*Il22<sup>-/-</sup>*) were donated by Pfizer (Cambridge, MA). All mice were maintained in specific pathogen-free facilities at the University of Pennsylvania. Prior to infection, mice were anesthetized using a ketamine and xylazine mixture and monitored until mice were fully awake. At the end of the experiments, mice were humanely euthanized using carbon dioxide inhalation. All procedures were performed in accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

### **Parasite and infection**

*L. major* (WHO /MHOM/IL/80/Friedlin wild-type *L. major*) and *L. braziliensis* (MHOM/BR/01/BA788) (de Moura et al. 2005) promastigotes were grown to the stationary phase in Schneider's Drosophila medium (GIBCO BRL,

Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Invitrogen USA), 2 mM L-glutamine, 100 U of penicillin and 100 µg of streptomycin per mL. Infective-stage promastigotes (metacyclics) were isolated from 4-5 day old (*L. major*) and 7 day old (*L. braziliensis*) stationary culture by density gradient separation by Ficoll (Sigma) (Spath and Beverley 2001). Mice were inoculated intradermally in the ear with 10 µL of PBS containing  $2 \times 10^6$  *L. major* metacyclic promastigotes. In some experiments mice were infected with a low dose of parasite ( $2 \times 10^3$ ) or a super-high dose of parasites ( $2 \times 10^7$ ). Lesion development was measured weekly by ear thickness with a digital caliper (Fisher Scientific). Mice were also assessed for pathology, using the following score system: no lesion (0), swelling/redness (1), deformation of the ear pinna (2), ulceration (3), partial tissue loss (4), and total tissue loss (5). Parasite burden in lesion tissues was assessed using a limiting dilution assay as previously described (Zaph et al. 2004). Freeze-thawed antigen (FTAg) was obtained from stationary-phase promastigotes of *L. major*. Soluble leishmanial antigen (SLA) was prepared from *L. braziliensis* parasites as previously described (Reed et al. 1986).

### **Patients and recall assays**

All cutaneous leishmaniasis patients were seen at the health post in Corte de Pedra, Bahia, Brazil, which is a well-known area of *L. braziliensis* transmission. The criteria for diagnosis were a clinical picture characteristic of



cutaneous leishmaniasis in conjunction with parasite isolation or a positive delayed-type hypersensitivity response to *Leishmania* antigen, plus histological features of cutaneous leishmaniasis. In all cases, the immunological analysis was performed before therapy. For cell culture and IL-22 measurement, peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood layered over a Ficoll-Hypaque gradient (GE Healthcare), then washed and resuspended in RPMI1640 medium with 10% heat inactivated human AB serum (Sigma) at a concentration of  $3 \times 10^6$  cells/mL. These cells were added to 24-well plates and were kept unstimulated or were stimulated with soluble leishmania antigen (5 ug/mL) for 96 h at 37C in 5% CO<sub>2</sub>. The supernatants were collected and stored frozen until analyzed for cytokines. IL-22 was measured by enzyme-linked immunosorbent assay (Pfizer).

### **Preparation of dermal sheets**

The dorsal and ventral sides of the mouse ear were split mechanically and placed dermis side down in a 24 wells plate in RPMI 1640 containing 0.25 mg/mL of Liberase TL (Roche, Diagnostics Corp.) and 10 mg/mL DNase I (Sigma-Aldrich). Ears were incubated for 90 min at 37° C in a 24-well plate. Dermal cell suspensions were prepared by dissociation on 70- um cell strainer (Falcon) in PBS containing 0.05% BSA and 20 mM EDTA.

### ***In vitro* restimulation and cytokine measurements**

For measurements of antigen-specific cytokine production in the mouse, the retroauricular lymph node was removed, mechanically dissociated, and single cell suspensions were prepared. Cells were resuspended in RPMI 1640 supplemented with 10% of FBS, 2 mM L-glutamine, 100 U of penicillin and 100 µg of streptomycin per mL and 0.05 mM of β-mercaptoethanol.  $4 \times 10^6$  cells per mL were plated in 24-well plates. Cells were incubated at 37°C in 5% CO<sub>2</sub> with  $20 \times 10^6$  *L. major* or *L. braziliensis* FTA<sub>g</sub>/mL. Supernatants were harvested 72 h after stimulation and assayed using a sandwich enzyme-linked immunosorbent assay (ELISA) for IFN-γ (eBioscience), IL-17 (eBioscience), and IL-22 (Pfizer). Cytokine concentrations were calculated from standard curves with a detection limit of 0.030 ng/mL.

### **Antibodies and flow cytometry**

Single cell suspensions from the ear were obtained as described above. For analysis of surface markers and intracellular cytokines, some cells were incubated for 4 h with 10 mg/mL of brefeldin A, 50 ng/mL of PMA and 500 ng/mL ionomycin (Sigma-Aldrich). Before staining, cells were incubated with an anti-Fcγ III/II receptor and 10% rat-IgG in PBS containing 0.1% BSA. Cells were stained for dead cells (Invitrogen) and surface markers (CD4, CD8b [BioLegend], CD45, Ly6G, CD11b [eBioscience]) followed by fixation with 2% of formaldehyde.

The data were collected using LSRII flow cytometer (BD) and analyzed using FlowJo software (Tree Star).

### **RNA isolation, purification, and quantitative real-time PCR**

Total RNA was extracted from ear tissue samples in 700uL of RLT lysis buffer (Qiagen). The sample was homogenized using a tissue homogenizer (FastPrep-24, MP Biomedical), and total RNA was extracted according to the recommendations of the manufacturer and further purified using the RNeasy Mini kit (QIAGEN). RNA was reverse transcribed using high capacity cDNA Reverse Transcription (Applied Biosystems). Real-time RT-PCR was performed on a ViiA™ 7 Real-Time PCR System (Applied Biosystems). Relative quantities of mRNA for several genes was determined using SYBR Green PCR Master Mix (Applied Biosystems) and by the comparative threshold cycle method, as described by the manufacturer. mRNA levels for each sample were normalized to Ribosomal protein S11 gene (RPS11). Primers were designed using Primer Express software (version 2.0; Applied Biosystems); *Rps11*, forward, 5'-CGTGACGAAGATGAAGATGC-3' and reverse, 5'-GCACATTGAATCGCACAGTC-3'; *Krt5*, forward, 5'-TTTGCCTCCTTCATCGACA-3' and reverse, 5'-CGGATCCAGGTTCTGCTTTA-3'; *Krt14*, forward, 5'-ATCGAGGACCTGAAGAGCAA-3' and reverse, 5'-TCGATCTGCAGGAGGACATT-3'; *Krt6a*, forward, 5'-GAGGAGAGGGAGCAGATCAA-3' and reverse, 5'-

CACTTGGTGTCCAGGACCTT-3'; *Krt16*, forward, 5'-  
TTGAGGACCTGAAGAGCAAGA-3' and reverse, 5'-  
CCTGGCATTGTCAATCTGC-3'; *II22*, 5'-ATGAGTTTTTCCCTTATGGGGAC-3'  
and reverse, 5'-GCTGGAAGTTGGACACCTCAA-3'; *II22bp*, forward, 5'-  
TCAGCAGCAAAGACAGAAGAAAC-3' and reverse, 5'-  
GTGTCTCCAGCCCAACTCTCA-3'; *lfng*, forward, 5'-  
GACTGTGATTGCGGGGTTGT-3' and reverse, 5'-  
GGCCCGGAGTGTAGACATCT-3'; *II4*, forward, 5'-  
ATGGAGCTGCAGAGACTCTT-3' and reverse, 5'-  
AAAGCATGGTGGCTCAGTAC-3'; *II17*, forward, 5'-  
CATGAGTCCAGGGAGAGCTT-3' and reverse, 5'-  
GCTGAGCTTTGAGGGATGAT-3'; *II12p40*, forward, 5'-  
TTGAAAGGCTGGGTATCGGT-3' and reverse, 5'-  
GAATTTCTGTGTGGCACTGG-3', *Tnfa*, forward, 5'-  
TCACTGGAGCCTCGAATGTC-3' and reverse, 5'-  
GTGAGGAAGGCTGTGCATTG-3'; *II6*, forward, 5'-  
ACAGAAGGAGTGGCTAAGGA-3' and reverse, 5'-CACCATGGAGCAGCTCAG-  
3'; *II10*, forward, 5'-TGTCCAGCTGGTCCTTTGTT-3' and reverse, 5'-  
ACTGCACCCACTTCCCAGT-3'; *Tgfb*, forward, 5'-  
CGCTGCTACTGCAAGTCAGA-3' and reverse, 5'-  
GGTAGCGATCGAGTGTCCA-3'; *II27p28*, forward, 5'-  
GATTGCCAGGAGTGAACCTG-3' and reverse, 5'-  
CGAGGAAGCAGAGTCTCTCAG-3'; *II1a*, forward, 5'-

TTGGTTAAATGACCTGCAACA-3' and reverse, 5'-  
GAGCGCTCACGAACAGTTG-3'; //1b, forward, 5'-TTGACGGACCCCAAAAGAT-  
3' and reverse, 5'- GATGTGCTGCTGCGAGATT-3'.

### **Microbiota collection, sequencing, and analysis**

Two independent experiments were performed using littermates as controls, with n=9-10 mice per cohort for a total of 9 //22<sup>-/-</sup> mice, 3 //22<sup>+/-</sup> mice, and 7 //22<sup>+/+</sup> mice. Microbiota was collected from the ear of the mouse using a swab (Catch-all Sample Collection Swab, Epicentre) moistened in Yeast Cell Lysis Buffer (from MasterPure Yeast DNA Purification Kit; Epicentre). DNA was isolated from swab specimens and amplification of the 16S-V4 region was performed as previously described (Hannigan et al. 2014). Sequencing of 16S rRNA amplicons was performed at the Penn Next Generation Sequencing Core using the Illumina MiSeq platform with 150 bp paired-end 'V2' chemistry.

### **Pre-processing and community characterization of 16S rRNA gene sequence data**

Sequence pre-processing followed methods previously described (Hannigan et al. 2014), but modified by subsampling at 11,000 sequences per sample. QIIME 1.6.0 (Caporaso et al. 2010) was used for initial stages of sequence analysis. Sequences were clustered into OTUs (operational taxonomic

units, a proxy for 'species') using UCLUST(Edgar 2010) at 97% sequence similarity. Bacterial diversity was calculated using the following alpha diversity indices: 1) Shannon diversity index; 2) Faith's phylogenetic distance (PD); and 3) Chao I species estimation; and 4) number of observed OTUs. Relative abundance of bacteria was calculated based on taxonomic classification of sequences using the RDP classifier (Wang et al. 2007) at a confidence threshold of 0.8. Microbiota data was analyzed with the R statistical software environment ([www.r-project.org](http://www.r-project.org)). Statistical significance was determined using two-sample Wilcoxon tests and corrected for multiple comparisons by FDR where appropriate.

## **Statistical analysis**

Results represent means  $\pm$  SEM. Data were analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined by one-way ANOVA when comparing more than two groups and by an unpaired two-tailed Student's t test to compare means of lesion sizes, parasite burdens, and cytokine production from different groups of mice. Statistically significant differences were defined as \* when  $p$  values  $<0.05$ .

## **2.4 Results**

### **Leishmania infections induce the production of IL-22**

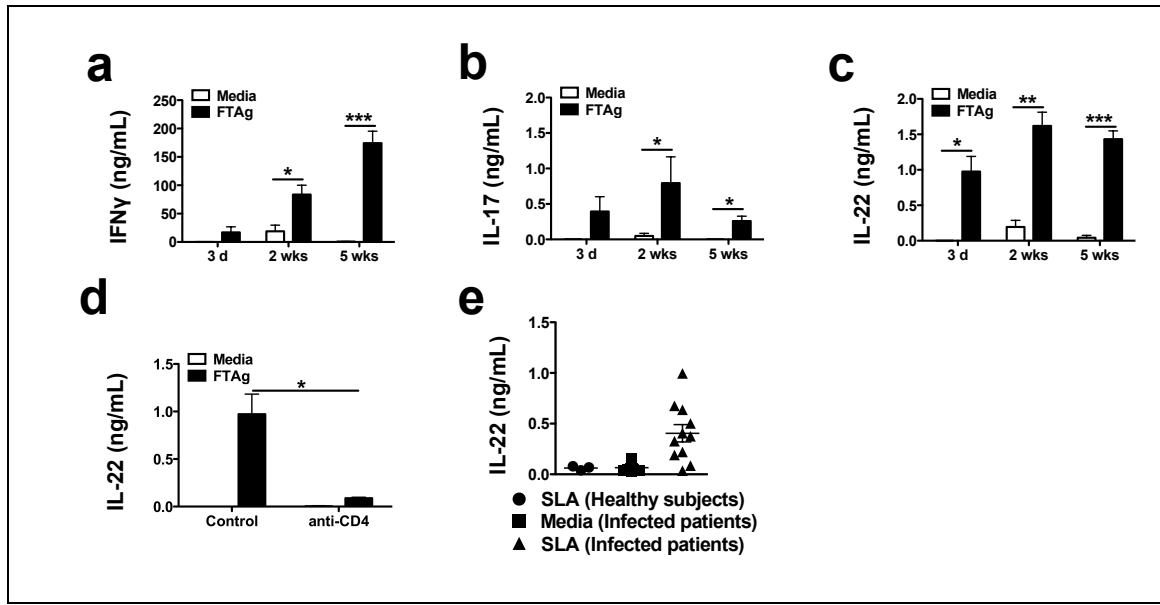
Since IL-22 can have tissue protective effects, we investigated whether IL-22 might help control pathology during infection with leishmania. We first asked whether infection with leishmania parasites led to an increase in IL-22 production. C57BL/6 (wild-type) mice were infected with *L. major* and were euthanized at 3 days, 2 weeks or 5 weeks after infection. Cells from the draining lymph nodes were stimulated with leishmanial antigen and cytokine levels were assessed. As expected during infection with *L. major*, IFN- $\gamma$  and IL-17 were produced in an antigen dependent manner (Figure 2a-b). As early as 3 days after infection there was an antigen specific production of IL-22, which was maintained at 2 and 5 weeks post-infection (Figure 2c). Because we know CD4<sup>+</sup> T cells can be a major source of IL-22 (Liang et al. 2006; Zheng et al. 2007), we wanted to determine if CD4<sup>+</sup> cells contributed to the antigen-specific production of IL-22 during *L. major* infection. Thus, C57BL/6 mice were infected with *L. major* and depleted of CD4<sup>+</sup> cells in vivo using a neutralizing antibody 2 days prior to sacrificing the mice. Cells were harvested from the draining lymph nodes at 3 days post-infection and cultured with media alone or with *L. major* antigen for 72 hours. Antigen stimulated cells from anti-CD4 treated mice produced significantly less IL-22 than untreated mice (Figure 2d), demonstrating that the production of IL-22 is dependent on the presence CD4<sup>+</sup> T cells. We also observed the production of IL-22 from cells of mice infected with another species of the parasite, *L. braziliensis* (data not shown). To determine if patients infected with *L. braziliensis* parasites also produced IL-22, peripheral blood mononuclear cells (PBMCs) from leishmaniasis patients were isolated and cultured with leishmanial

antigen. Similar to cells from mice, PBMCs from infected patients, but not healthy subjects, produced IL-22 in response to stimulation with leishmanial antigen (Figure 2e), suggesting that IL-22 may be important in human patients as well as in experimental murine infections.

### **IL-22 limits pathology during leishmania infection independent of parasite control**

To determine if IL-22 plays a protective role during the course of infection with leishmania, C57BL/6 and *IL22<sup>-/-</sup>* mice were infected with *L. major* and the disease monitored. *IL22<sup>-/-</sup>* mice exhibited larger lesions compared with wild-type mice (Figure 3a). We noticed that in addition to greater swelling, the ears of *IL22<sup>-/-</sup>* mice often exhibited more severe pathology than wild-type mice, and in some cases led to tissue loss at the site of infection (Figure 3b). To quantify these





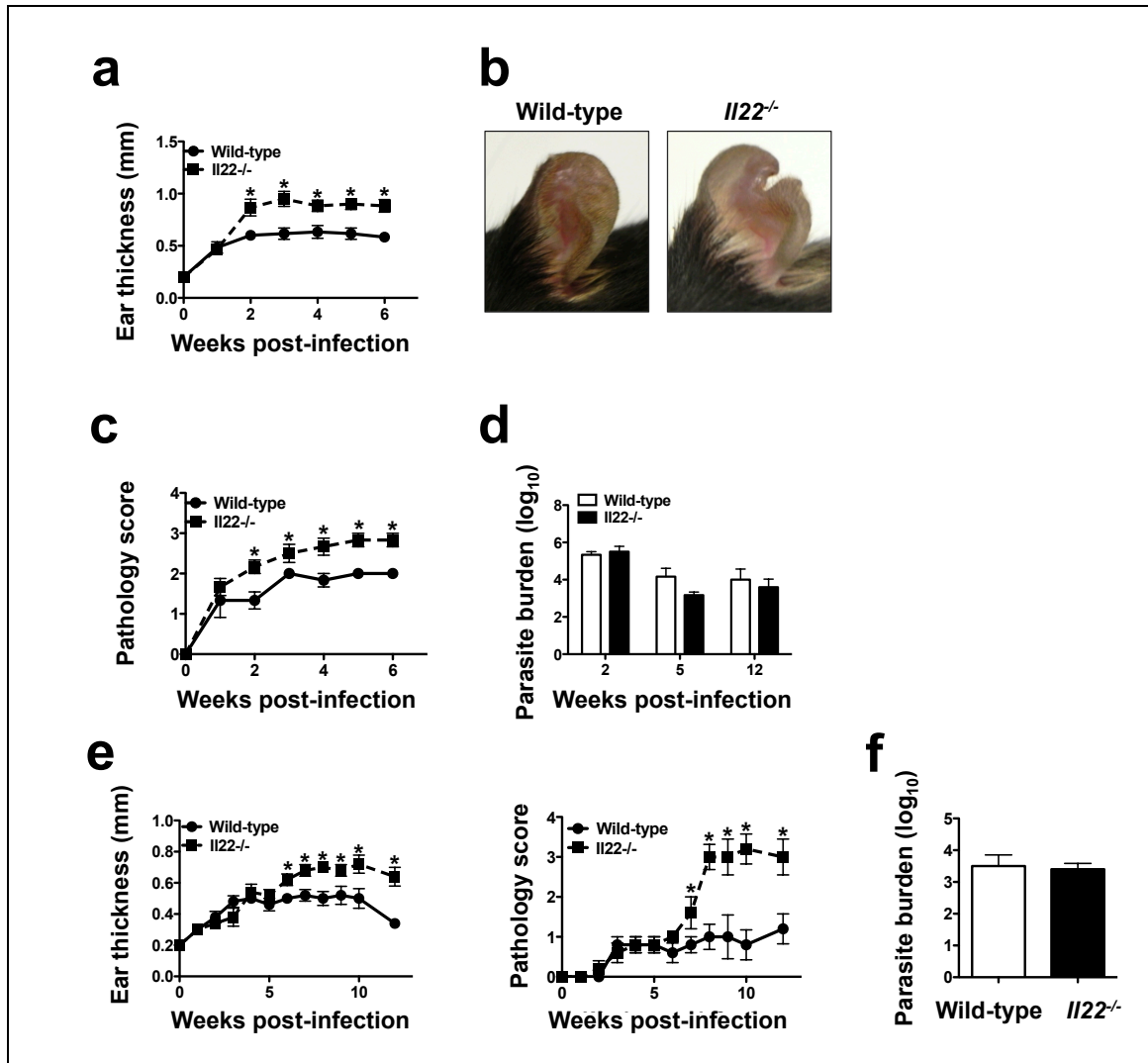
**Figure 2: IL-22 is induced during leishmania infections.** C57BL/6 mice were intradermally infected with  $2 \times 10^6$  *L. major* promastigotes metacyclics in the ear. Cells from the draining lymph nodes of infected mice were isolated and cultured for 72 hours with media or leishmania antigen. Supernatants were collected and (a) IFN- $\gamma$  (b) IL-17, and (c) IL-22 release was measured by ELISA. (d) C57BL/6 mice were intradermally infected with  $2 \times 10^6$  *L. major* promastigotes metacyclics in the ear and two days later treated with anti-CD4. Mice were euthanized on day 3 and cells from the draining lymph nodes were isolated and cultured for 72 hours with media or leishmania antigen to analyze IL-22 production by ELISA. (e) PBMCs from healthy subjects and *L. braziliensis* infected patients were cultured for 72 hours with media or *L. braziliensis* antigen. Supernatants were collected and analyzed for IL-22 release by ELISA. Data are representative of at least 3 independent experiments, with 3-5 mice per group. Error bars indicate mean  $\pm$  SEM, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

changes, we employed a scoring system that better captures the pathology associated with leishmania infection. As seen in Figure 3c, *IL22*<sup>-/-</sup> mice exhibited greater pathology than wild-type mice infected with *L. major*. To determine if the increased pathology observed following *L. major* infections was due to higher parasite levels in *IL22*<sup>-/-</sup> mice, we assessed the parasite burden in wild-type and *IL22*<sup>-/-</sup> mice at 2, 5 and 12 weeks of infection, and found no significant differences (Figure 3d).

*L. braziliensis* parasites are known to induce a particularly strong inflammatory response in patients, and also cause mucosal leishmaniasis, the most severe form of the disease (de Oliveira and Brodskyn 2012). Therefore, we asked if IL-22 regulated the lesion resolution in this infection as well. We infected wild-type and *IL22*<sup>-/-</sup> mice with *L. braziliensis* and followed the course of infection. As with *L. major*, *L. braziliensis* infected *IL22*<sup>-/-</sup> mice had significantly larger lesions than wild-type mice with more pathology, but no differences in the number of parasites within the lesions (Figure 3e-f).

### **IL-22 maintains wound-healing capabilities in the skin during *L. major* infection**

The resolution of a leishmanial lesion is analogous to wound healing, which requires keratinocyte proliferation and differentiation (Martin 1997). Therefore, we analyzed the expression of several genes at the peak of infection to assess keratinocyte functions in the lesions of wild-type and *IL22*<sup>-/-</sup> mice. We observed no difference in the expression of keratin 5 and keratin 14, both of which are expressed in proliferating keratinocytes, between wild-type and *IL22*<sup>-/-</sup> mice (Figure 4a). We then decided to look at other keratins, which are upregulated in chronic wounds and can inhibit the ability of keratinocytes to efficiently heal

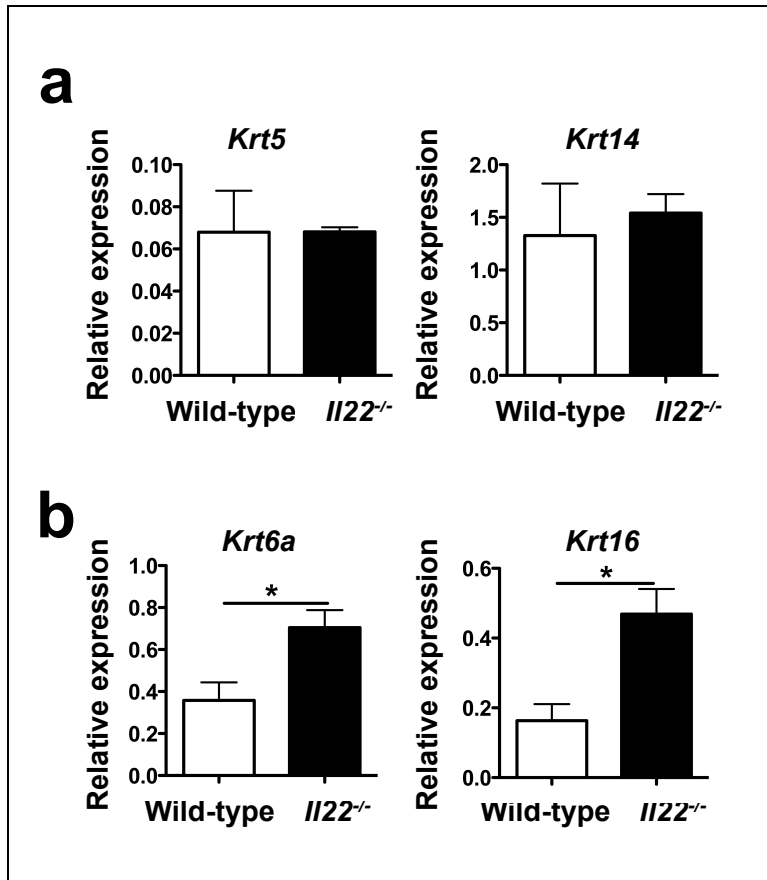


**Figure 3: IL-22 limits pathology during leishmania infection.** (a) C57BL/6 (wild-type) and *Il22<sup>-/-</sup>* mice were intradermally infected with  $2 \times 10^6$  *L. major* promastigote metacyclics and euthanized at various time-points after infection. The lesions were assessed by measuring ear thickness for 6 weeks. (b) Pictures were taken at 5 weeks post-infection. (c) Lesion pathology was determined based on a pathology score. (d) Number of parasites in the lesions was quantified using a limiting assay at 2, 5, and 12 weeks post-infection. (e) Wild-type and *Il22<sup>-/-</sup>* mice were intradermally infected with  $2 \times 10^6$  *L. braziliensis* promastigote metacyclics and lesions were assessed by measuring ear thickness and given a pathology score for 12 weeks and (f) parasite numbers were quantified using a limiting dilution assay in the lesions at 12 weeks post-infection. Data are representative of at least 2 independent experiments, with 3-5 mice per group. Error bars indicate mean  $\pm$  SEM, \* $p < 0.05$ .

wounds and damage (Rotty and Coulombe 2012; Wawersik et al. 2001). We observed that *IL22*<sup>-/-</sup> mice had higher expression of keratin 6a and keratin 16 (Figure 4b), both of which are known to inhibit keratinocytes migration. Thus, one role of IL-22 during cutaneous leishmaniasis may be to promote wound healing capabilities of keratinocytes by regulating the expression of keratins involved in migration and differentiation.

### **The requirement for IL-22 depends on parasite burden and inflammation**

Recently, it was reported that IL-22 does not play a role during a low dose of infection with *L. major* (Brosch et al. 2014). Our results, taken together with other findings prompted us to consider the possibility that IL-22 might only be required when a threshold of inflammation and tissue damage was present. To test this hypothesis, we infected mice with a super high dose of parasites ( $2 \times 10^7$ ), an intermediate dose ( $2 \times 10^6$ ), and with a low dose of parasites ( $2 \times 10^3$ ), and followed the course of infection. Because we noticed some variability between experiments, we decided to pool data from multiple experiments and compare pathology at the peak of infection. Similar to recent findings in which mice were infected with a low dose of parasites (Brosch et al. 2014), we observed no difference in the lesion size or pathology between wild-type and *IL22*<sup>-/-</sup> mice when infected with  $2 \times 10^3$  parasites (Figure 5a). On the other hand, *IL22*<sup>-/-</sup> mice



**Figure 4: IL-22 regulates the expression of skin repair genes during *L. major* infection.** (a-b) Wild-type and *IL22*<sup>-/-</sup> mice were intradermally infected with  $2 \times 10^6$  *L. major* promastigote metacyclics and RNA was isolated from the lesions at 5 weeks post-infection to assess gene expression. Data are represented as relative expression to housekeeping gene *rps11* and are representative of at least 2 independent experiments, with 3-5 mice per group. Error bars indicate mean  $\pm$  SEM, \* $p < 0.05$ .

infected with  $2 \times 10^6$  and  $2 \times 10^7$  parasites had more pathology than their wild-type counterparts (Figure 5b-c). We euthanized these animals at 5 weeks post-infection and assessed their parasite burdens. As expected from the results described above, no differences were observed in the parasite burden between wild-type and *IL22*<sup>-/-</sup> mice (data not shown). We then measured levels of IL-22 expression in the lesions, and found significantly higher expression of *IL22* mRNA

when mice were infected with more parasites (Figure 5d). The IL-22 binding protein (IL-22BP) is a soluble receptor that inhibits IL-22 signaling through its receptors (Xu et al. 2001). Thus, we examined the expression of *Il22bp* in wild-type mice infected with *L. major* infection. Unlike IL-22, IL-22BP was expressed at significantly lower levels when mice were infected with more parasites (Figure 5e). These results suggest that following infection with high numbers of parasites IL-22 is induced to a greater extent and less inhibited by IL-22BP, and that IL-22 helps regulate the pathology associated with a higher parasite burden.

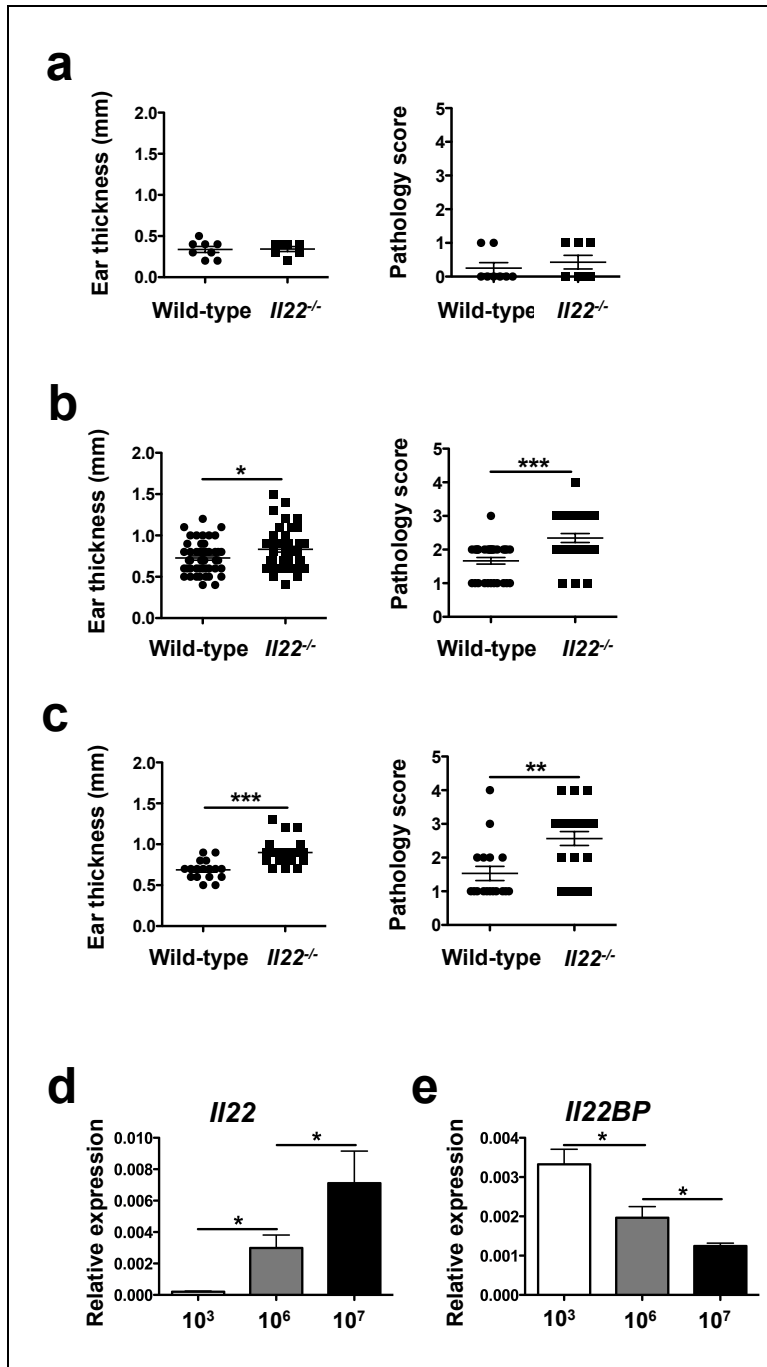
### **IL-22 does not modulate the skin microbiota at the steady state**

Recent studies indicate that the skin microbiota influences the pathology associated with leishmania infection (Naik et al. 2012). Since IL-22 regulates the production of antimicrobial peptides (AMPs) (Liang et al. 2006; Sonnenberg et al. 2012), we considered the possibility that homeostatic levels of IL-22 might influence AMP levels, resulting in changes in the skin microbiota and consequently disease development. To test this idea, the ears of uninfected *Il22<sup>+/+</sup>/Il22<sup>+/-</sup>* and *Il22<sup>-/-</sup>* littermates were swabbed to extract bacterial DNA. 16S ribosomal RNA genomic sequencing was performed and the skin microbiota was analyzed. In two independent experiments (n=9 *Il22<sup>-/-</sup>* mice and n=10 control littermate mice) no significant differences in bacterial diversity were observed between littermate controls and *Il22<sup>-/-</sup>* mice (Figure 6a). There were also no differences in the relative abundance of the bacterial communities between

controls and *Il22*<sup>-/-</sup> mice (Figure 6b). These findings indicate that *Il22*<sup>-/-</sup> mice do not have a dysbiotic skin microbiota responsible for the increased pathology.

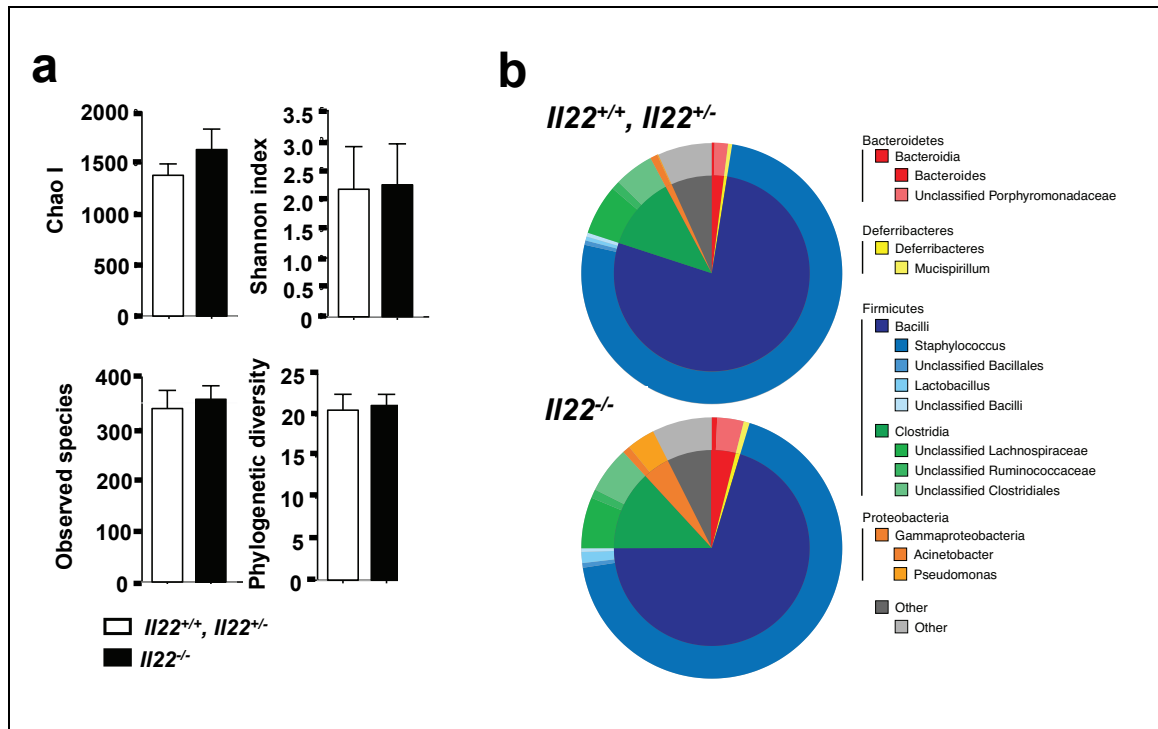
**IL-22 does not regulate inflammatory cell infiltrate, but rather limits tissue damage during *L. major* infection.**

Because we observed more pathology and inflammation in the *Il22*<sup>-/-</sup> mice, we wanted to determine if there was increased inflammatory cell infiltrate in the lesions of these mice. We examined the presence of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, CD11b<sup>+</sup> myeloid cells, and neutrophils in the lesions of *L. major* infected wild-type and *Il22*<sup>-/-</sup> mice at the peak of infection. While there was an increase over naïve skin in the frequency and numbers of T cells of wild-type and *Il22*<sup>-/-</sup> lesions and in the numbers of myeloid cells, there was no difference in these populations between wild-type and *Il22*<sup>-/-</sup> mice (Figure 7a). We also assessed transcript levels of inflammatory and regulatory cytokines in the lesions of wild-type and *Il22*<sup>-/-</sup> mice. As expected, *Ifng* levels were increased following infection, and there was a similar increase in wild-type and *Il22*<sup>-/-</sup> mice. There were minimal or no changes in *Il4*, *Il17*, *Tnfa*, *Il12a*, *Il6*, *Il10*, *Tgfb* and *Il27p28* gene expression between naïve skin and leishmanial lesions, and no significant differences between wild-type and *Il22*<sup>-/-</sup> mice (Figure 7b). However, we found that the lesions of *Il22*<sup>-/-</sup> mice had higher expression of *Il1a* and *Il1b* compared with wild-type mice (Figure 7c). The expression of these molecules is often observed in inflamed tissue and can be



**Figure 5: The requirement for IL-22 is parasite dose dependent.** Lesion sizes and pathology scores were compiled from several experiments at 5 weeks post-infection from wild-type and *II22*<sup>-/-</sup> mice that were intradermally infected with (a)  $2 \times 10^3$ , (b)  $2 \times 10^6$ , or (c)  $2 \times 10^7$  *L. major* metacyclics. RNA was isolated from the lesions of wild-type mice infected with *L. major* to assess (d) *II22* and (e) *II22BP* expression. Data are represented as relative expression to housekeeping gene *rps11* and are representative of at least 2 independent experiments, with 3-5 mice per group. Error bars indicate mean  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .





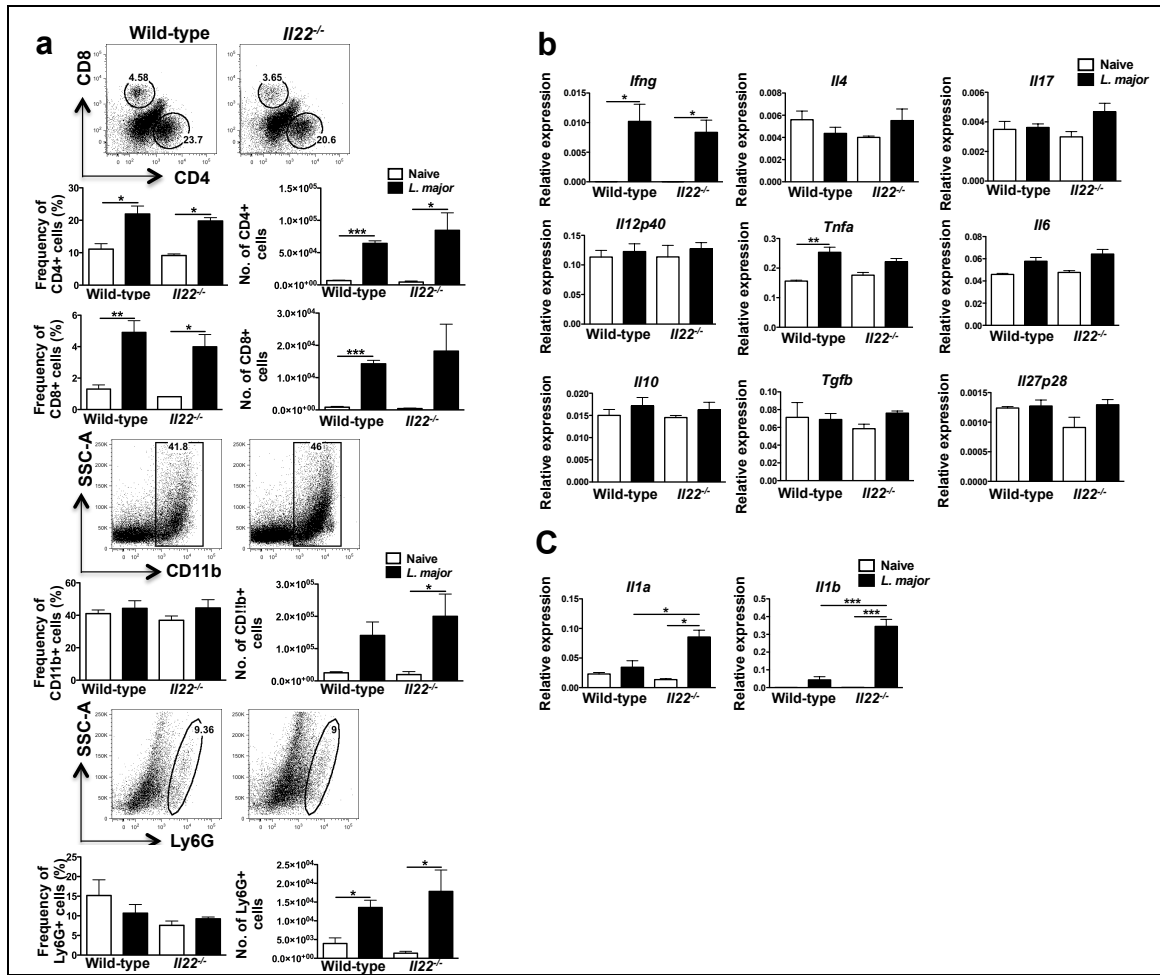
**Figure 6: IL-22 does not modulate the skin microbiota at the steady state.** Swabs were collected from *II22<sup>+/+</sup>*, *II22<sup>+/-</sup>*, and *II22<sup>-/-</sup>* cohoused littermates and bacterial DNA was isolated and sequenced. **(a)** Within sample diversity was calculated using four commonly utilized alpha metrics: Chao I, Shannon Index, Observed Species, and Faith's Phylogenetic Diversity. **(b)** The microbiota composition was calculated at multiple phylogenetic levels. The outer ring represents the relative contributions of the 12 most prevalent genera. The inner ring represents the corresponding class for each genus. The remaining genera were compiled into the "Other" category depicted in gray. Data are representative of 2 independent experiments, with 4-5 mice per group.

induced and released when cells encounter tissue damage (Carta, Lavieri, Rubartelli 2013). Although there were no differences in the immune response between wild-type and *II22<sup>-/-</sup>* mice, increased expression of these damage-associated molecules demonstrates that *II22<sup>-/-</sup>* mice directly or indirectly regulate their production during infection with *L. major*.

## 2.5 Discussion

Our results uncover a previously unknown role for IL-22 during cutaneous leishmaniasis. While a pathologic and inflammatory role for IL-22 has been reported in other cutaneous diseases (Ma et al. 2008; Pantelyushin et al. 2012; Van Belle et al. 2012), we found that IL-22 does not promote increased inflammation during infection with *Leishmania spp.* Rather, *IL22<sup>-/-</sup>* mice exhibited more tissue damage than wild-type mice when infected with *L. major* or *L. braziliensis*, suggesting that IL-22 limits pathology when a threshold of inflammation is reached during leishmaniasis.

Our results demonstrate that the production of IL-22 is dependent on the presence of CD4<sup>+</sup> T cells, which have previously been shown to produce IL-22 (Liang et al. 2006; Zheng et al. 2007). However,  $\gamma\delta$  T cells, NK cells, ILCs and neutrophils are other potential sources of IL-22 that might contribute to the IL-22 observed in these lesions (Carlsen et al. 2015; Taube et al. 2011; Van Belle et al. 2012; Xu et al. 2014). Interestingly, the production of IL-22 appeared to be dose-dependent, such that mice infected with higher doses of *L. major* expressed higher levels of IL-22 in the lesions. Inflammation and damage in other models of disease have been shown to induce IL-22 expression (Aujla and Kolls 2009; Sonnenberg et al. 2010; Zenewicz et al. 2008; Zheng et al. 2007; Zheng et al. 2008), consistent with our findings that higher doses of *L. major* elicit more



**Figure 7: IL-22 does not alter the immune response during *L. major* infection.** Wild-type and *Il22*<sup>-/-</sup> mice were intradermally infected with 2 × 10<sup>6</sup> *L. major* promastigote metacyclics and cells from 5 week old lesions were collected and analyzed by flow cytometry. **(a)** Representative dot plots and bar graphs depict frequencies and total cell numbers of CD4+, CD8+, CD11b+, and LY6G+ cells. **(b-c)** RNA was isolated from the lesions of wild-type mice infected with *L. major* to assess gene expression. Data are represented as relative expression to housekeeping gene *rps11* and are representative of at least 3 independent experiments, with 3-5 mice per group. Error bars indicate mean ± SEM, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

inflammation and higher expression of IL-22. Conversely, we observed a decrease in the expression of the IL-22 antagonist, IL-22BP, in mice with higher doses of the parasite. This inverse relationship of IL-22/IL-22BP regulating tissue damage has also been observed during Hepatitis C and schistosome infections

(Sertorio et al. 2015). Thus, we hypothesize that having a high IL-22/IL-22BP ratio is required to limit pathology. In order to determine whether the immune response was influenced by the absence of IL-22, we assessed cytokine responses within leishmanial lesions of *Il22<sup>-/-</sup>* mice. Changes in the balance of Th1 and Th2 cytokines is often associated with increased susceptibility to *L. major*, but since there were no differences in the parasite burden it was not surprising that the mRNA levels of *Ifng*, *Tnfa*, *Il12p40* and *Il4* were similar in both wild-type and *Il22<sup>-/-</sup>* mice. Moreover, there were no differences in the cellular infiltrate of T cells and myeloid cells in the lesions of wild-type and *Il22<sup>-/-</sup>* mice. These results prompted us to consider other ways in which IL-22 can provide tissue protection during inflammation.

*L. major* infection leads to the development of ulcerated lesions that eventually resolve due to tissue remodeling at the infection site (Baldwin et al. 2007; Elso et al. 2004a; Elso et al. 2004b). IL-22 promotes wound healing by increasing epithelial cell proliferation, decreasing the differentiation of keratinocytes and inducing anti-apoptotic molecules in keratinocytes (Boniface et al. 2005; Radaeva et al. 2004; Vogl et al. 2004; Wolk et al. 2006). Thus, one way IL-22 may enhance wound healing in leishmaniasis is by regulating *L. major* induced keratinocyte death. Additionally, IL-22 stimulates fibroblasts to produce extracellular matrix proteins, as well as increases the differentiation of myofibroblasts that help to contract wounds (McGee et al. 2013), and both of these functions could be critical in the resolution of leishmanial lesions. In this study, we found another mechanism in which IL-22 contributes to wound healing

and tissue repair. Keratinocyte proliferation and differentiation are critically regulated processes during wound repair (Martin 1997). Upon injury, activated keratinocytes migrate to close the wound, while basal keratinocytes proliferate at the basement membrane (Usui et al. 2005; Usui et al. 2008). In order for a cell to proliferate and repair the basement membrane, differentiation must be halted (Usui et al. 2005). IL-22 can induce proliferation, but also down-regulate keratinocyte differentiation and keratin expression (Boniface et al. 2005). Thus, we decided to examine the expression of various proliferation and differentiation markers. While the proliferation markers, keratin 5 and keratin 14 were unaffected by the absence of IL-22, the lesions of *IL22<sup>-/-</sup>* mice expressed higher levels of keratins 6a and 16. These genes are induced in keratinocytes upon injury and are maintained during reepithelialization. However, the intensity in expression levels of these keratins is important because their overexpression can lead to defects in keratinocyte migration and wound closure (Wawersik et al. 2001). The higher expression of keratins 6a and 16 observed in chronic wounds is consistent with our data showing that *IL22<sup>-/-</sup>* mice have a defect in wound repair during *L. major* infection. Interestingly, lower expression of keratin 16 or deletion of keratin 6a can enhance keratinocyte migration (Rotty and Coulombe 2012), which may explain the eventual lesion resolution in wild-type mice with lower expression of these keratins. Keratinocyte differentiation and migration are key to wound healing, and thus our results suggest that IL-22 may be important in regulating these processes through keratins 6a and 16 in order to efficiently resolve leishmanial lesions.

While IL-22 protects against certain pathogens, such as *Klebsiella pneumoniae* and *Citrobacter rodentium* (Aujla et al. 2008; Zheng et al. 2008), in our study we found no evidence that IL-22 contributes to control of *L. major* or *L. braziliensis*, as wild-type and *Il22<sup>-/-</sup>* mice contained the same number of parasites in their lesions. These results are similar to those observed with other parasites, such as *Toxoplasma gondii* or *Schistosoma mansoni* (Wilson et al. 2010). However, this is in contrast to visceral leishmaniasis, where the production of IL-22 has been correlated with increased protection (Ghosh et al. 2013; Pitta et al. 2009). How IL-22 promotes resistance in visceral leishmaniasis is unknown, but it is unlikely to be a direct effect on the parasites, since the IL-22R is not expressed on the cells infected with leishmania (Wolk et al. 2004). Since stromal cells play a role in immunoregulation in visceral leishmaniasis, one possibility is that stimulation of stromal cells by IL-22 might indirectly influence the development of disease (Svensson et al. 2004).

IL-22 helps maintain barrier function in the skin, but when produced at high levels and/or in the context of other proinflammatory cytokines, such as IL-17, IL-22 promotes increased pathology (Sonnenberg et al. 2010). The factors that determine whether IL-22 will play a protective or pathologic role remain poorly understood, although it has been suggested that the nature of the inflammatory response may be a determining factor (Sonnenberg et al. 2010). Our results indicate that one factor determining whether IL-22 is important in protection in the skin may be the degree of damage induced. Thus, when *Il22<sup>-/-</sup>* mice were infected with a high dose of parasites, we routinely saw increased

pathology in *IL22*<sup>-/-</sup> mice compared with wild-type mice, while we found no differences in the development of lesions in *IL22*<sup>-/-</sup> mice and wild-type mice when the animals were infected with a low dose of parasites. The latter finding would account for the results of a prior study where IL-22 was reported to have no role in *L. major* infection (Brosch et al. 2014). These results suggest that the protective role for IL-22 requires a threshold of inflammation that is reached at high parasite doses in this experimental model. This raises the issue of how our murine studies relate to human leishmaniasis. While the initial dose of parasites transmitted by sandflies is much less than the high doses we have studied here, patients also exhibit significantly more pathology than what occurs in low dose infections in mice. Thus, we hypothesize that in more severe forms of cutaneous leishmaniasis, as often seen following *L. braziliensis* infection, IL-22 might be induced to ensure that even more severe disease does not develop. Consistent with this was our finding that cells from patients made IL-22 in response to stimulation, indicating that there was sufficient damage in the patients to promote IL-22 production.

Taken together, our results in *IL22*<sup>-/-</sup> mice show that IL-22 limits pathology during cutaneous leishmaniasis and suggest that once a certain threshold of damage is reached, IL-22 is expressed at higher levels and limits subsequent damage by maintaining skin barrier integrity and wound healing capacities. In the absence of IL-22, not only do lesions fail to resolve, but higher expression of the inflammatory molecules IL-1 $\alpha$  and IL-1 $\beta$  may lead to even greater tissue destruction. Thus, IL-22 plays an important, and previously unappreciated, role in

maintaining skin repair properties and limiting inflammation during cutaneous leishmanial infections.



## CHAPTER 3: CUTANEOUS LEISHMANIASIS INDUCES A TRANSMISSIBLE DYSBIOTIC SKIN MICROBIOTA THAT PROMOTES SKIN INFLAMMATION

### 3.1 Abstract

Skin microbiota can impact allergic and autoimmune responses, wound healing and defense against pathogens. Here, we investigated their role in cutaneous leishmaniasis. We found that infection with leishmania altered the skin microbiota at the lesion site, characterized by increases in the abundance of *Staphylococcus*, *Streptococcus*, or both. When we infected mice with leishmania we observed similar changes depending upon disease severity. Unexpectedly, the dysbiosis was not limited to the lesion site, but was transmissible to skin distant from the infection site, and to skin from co-housed naïve mice. This observation allowed us to test whether a pre-existing dysbiotic skin microbiota influences disease. We found that dysbiotic naïve mice challenged with *L. major* or tested for contact hypersensitivity had exacerbated skin inflammatory responses. These findings demonstrate that a dysbiotic skin microbiota is not only a consequence of skin injury, but also enhances inflammation, which has implications for many inflammatory cutaneous diseases.

### 3.2 Introduction

The skin is a barrier and the body's first line of defense against injury and infection. It also hosts commensal populations of bacteria, fungi and viruses that may influence wound healing, the immune response to infection, and inflammatory responses that occur in chronic diseases(Canesso et al. 2014; Grice et al. 2010; Naik et al. 2012). Though there are strong associations between certain human diseases and changes in the skin microbiota(Kong et al. 2012; Loesche et al. 2016; Oh et al. 2013), the consequences of such changes are unclear, including the role of skin commensal microbes in modulating dermal cellular responses. Animal models in which microbial communities can be manipulated are essential to determine whether these changes influence the outcome of disease.

Cutaneous leishmaniasis is caused by intracellular protozoan parasites and is characterized by a spectrum of clinical manifestations, ranging from self-healing single lesions to chronic, and in some cases metastatic, lesions(Scott and Novais 2016). The factors responsible for chronic disease in leishmaniasis are still being defined, although it is clear that some of the most severe forms of the disease are not caused by uncontrolled parasite replication, but rather an exaggerated immune response leading to excessive inflammation(Antonelli et al. 2005; Lopez Kostka et al. 2009; Santos Cda et al. 2013; Gonzalez-Lombana et al. 2013; Novais et al. 2013; Crosby et al. 2014). Unfortunately, there is no

vaccine for leishmaniasis and drug treatment is often ineffective, which provides the impetus for better understanding the factors that drive the destructive inflammatory responses. Some of these severe forms of disease can be mimicked in mice, which can develop healing or non-healing disease following *L. major* infection depending upon whether a dominant Th1 or Th2 response develops (Scott and Novais 2016). Less well understood is the role the skin microbiota plays in cutaneous leishmaniasis. Although it has been reported that the course of infection in germ free mice differs from conventional mice (de Oliveira et al. 1999; Naik et al. 2012; Oliveira et al. 2005), how the skin microbiota changes in patients and conventional mice, and whether such changes influence disease is less clear.

In this study, we found that infection with leishmania parasites causes a decrease in bacterial diversity in the skin that is characterized by communities dominated by *Staphylococcus* spp. and/or *Streptococcus* spp in both humans and mice. We hypothesized that disease-associated shifts in the skin microbiota (“dysbiosis”) contribute to lesion pathology and dermal cellular responses, including immune and inflammatory responses in *L. major* infection. To test this we utilized a mouse model of cutaneous leishmaniasis, and found that infection with *L. major* changed the skin microbiota in a manner dependent on disease severity. Leishmania-induced dysbiosis was not confined to the site of infection, but occurred globally on the skin of infected mice, and moreover, was transferred to uninfected co-housed mice. Colonization of skin with *Staphylococcus xylosus* isolated from the dysbiotic mice increased inflammatory responses in a contact

hypersensitivity model, although not in normal skin, indicating that dysbiosis might exacerbate disease. Dysbiotic microbiota, when transferred to naïve mice prior to leishmania infection, increased disease pathology compared to control animals. Taken together these results indicate that the skin microbiota influences the inflammatory response in leishmaniasis and other inflammatory skin conditions. This work has significant implications for the treatment of cutaneous leishmaniasis and other skin diseases, and highlights the potential of the skin microbiota as a therapeutic target.

### **3.3 Materials and methods**

#### **Experimental model and subject details**

##### **Mice**

Female C57BL/6 and BALB/c mice 6-8 weeks old were purchased from the Charles River Laboratories (Durham, NC). All mice were maintained in specific pathogen-free facilities at the University of Pennsylvania. Cages were changed twice per week with glove changes between handling each cage. Unless stated otherwise, a minimum of 5 mice were used based on variability observed in previous experiments with *L. major*. Mice were randomly assigned to experimental groups by investigators. Investigators were not blinded in this study. Prior to infection, mice were anesthetized using a ketamine and xylazine mixture and monitored until the mice were fully awake. At the end of the experiments, mice were humanely euthanized using carbon dioxide inhalation. All procedures involving mice were performed in accordance with the guidelines of the

University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

### **Human Cutaneous Leishmaniasis Subjects**

All cutaneous leishmaniasis patients were seen at the health post in Corte de Pedra, Bahia, Brazil, which is a well-known area of *L. braziliensis* transmission. The criteria for diagnosis were a clinical picture characteristic of cutaneous leishmaniasis in conjunction with documentation of DNA of *L. braziliensis* by PCR, or parasite isolation or documentation of amastigotes in lesion biopsies by histopathology. In all cases, swabs were collected before therapy. There were 44 patients, both male (72.7%) and female (27.3%), with a median age of 27 years. This study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines (Ethical Committee of the Maternidade Climerio de Oliveira, Salvador, Bahia, Brazil; and the University of Pennsylvania Institutional Review Board). This study was approved by the Ethical Committee of the Federal University of Bahia (Salvador, Bahia, Brazil)(010/10) and the University of Pennsylvania IRB (Philadelphia, PA)(813390). All patients provided written informed consent for the collection of samples and subsequent analysis.

### **Parasite and Bacterial Cultures**

*L. major* (WHO/MHOM/IL/80/Friedlin wild-type *L. major*) promastigotes were grown to the stationary phase in Schneider's *Drosophila* medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Invitrogen USA), 2 mM L-glutamine, 100 U of penicillin and 100 µg of streptomycin per mL. Infective-stage promastigotes (metacyclics) were isolated from 4-5 day old (*L. major*)

## **Mice**

Female C57BL/6 and BALB/c mice 6-8 weeks old were purchased from the Charles River Laboratories (Durham, NC). All mice were maintained in specific pathogen-free facilities at the University of Pennsylvania. Cages were changed twice per week with glove changes between handling each cage. Unless stated otherwise, a minimum of 5 mice were used based on variability observed in previous experiments with *L. major*. Mice were randomly assigned to experimental groups by investigators. Investigators were not blinded in this study. Prior to infection, mice were anesthetized using a ketamine and xylazine mixture and monitored until the mice were fully awake. At the end of the experiments, mice were humanely euthanized using carbon dioxide inhalation. All procedures involving mice were performed in accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

## Human Cutaneous Leishmaniasis Subjects

All cutaneous leishmaniasis patients were seen at the health post in Corte de Pedra, Bahia, Brazil, which is a well-known area of *L. braziliensis* transmission. The criteria for diagnosis were a clinical picture characteristic of cutaneous leishmaniasis in conjunction with documentation of DNA of *L. braziliensis* by PCR, or parasite isolation or documentation of amastigotes in lesion biopsies by histopathology. In all cases, swabs were collected before therapy. There were 44 patients, both male (72.7%) and female (27.3%), with a median age of 27 years. This study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines (Ethical Committee of the Maternidade Climerio de Oliveira, Salvador, Bahia, Brazil; and the University of Pennsylvania Institutional Review Board). This study was approved by the Ethical Committee of the Federal University of Bahia (Salvador, Bahia, Brazil)(010/10) and the University of Pennsylvania IRB (Philadelphia, PA)(813390). All patients provided written informed consent for the collection of samples and subsequent analysis.

## Parasite and Bacterial Cultures

*L. major* (WHO/MHOM/IL/80/Friedlin wild-type *L. major*) promastigotes were grown to the stationary phase in Schneider's *Drosophila* medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Invitrogen USA), 2 mM L-glutamine, 100 U of penicillin and 100 µg

of streptomycin per mL. Infective-stage promastigotes (metacyclics) were isolated from 4-5 day old (*L. major*) stationary culture by density gradient separation by Ficoll (Sigma) (Spath and Beverley 2001). An isolate of *S. xylosus* and alpha-hemolytic *Streptococcus* was cultured from the ears of *L. major* infected mice. For topical associations and infections, the bacteria was cultured in Brain heart infusion (BHI) media (Remel, Lenexa, KS, USA) shaking for 12 hours at 37°C.

## Method details

### Leishmania infection and in vivo antibody depletions

Mice were inoculated intradermally in the ear with 10 µL of PBS containing  $2 \times 10^6$  *L. major* metacyclic promastigotes. Lesion development was measured weekly by ear thickness with a digital caliper (Fisher Scientific). Mice were also assessed for pathology, using the following score system: no lesion (0), swelling/redness (1), deformation of the ear pinna (2), ulceration (3), partial tissue loss (4), and total tissue loss (5). Parasite burden in lesion tissues was assessed using a limiting dilution assay as previously described (Zaph et al. 2004). In specified experiments, mice were treated with 500µg of anti-IL-12 mAb (BioXcell, clone R1-5D9) one day prior to infection and then twice per week for the duration of the experiment. Equal amounts of an isotype control, Rat IgG2a



(BioXcell, clone 2A3) was given in all experiments using in vivo antibody treatments.

### **Bacterial topical associations, intradermal infections, and CFU quantification**

For topical associations,  $10^8$ - $10^9$  CFUs of bacteria were applied to the entire mouse body using sterile cotton swabs, every other day for a total of 4 times. For intradermal infections, mice were inoculated with  $10\mu\text{L}$  of  $10^8$ - $10^9$  CFU bacteria/mL culture. For CFU quantification, the dermal sheets of the mouse ears were homogenized in 1mL of PBS using a tissue homogenizer (FastPrep-24, MP Biomedical) and plated on tryptic soy blood agar (Remel) or mannitol salt agar (Acumedia) in serial dilutions. Plates were incubated overnight at  $37^\circ\text{C}$  and CFUs were counted the next day.

### **Contact hypersensitivity and antibody treatments**

For sensitization, 1-fluoro-2,4-dinitrobenzene (DNFB) (Sigma-Aldrich) was added to a 3:1 acetone:olive oil dissolvent to get a final concentration of 0.5%. Mice were treated on the belly with  $30\mu\text{L}$  of the mixture. During the challenge phase, mice were treated with  $20\mu\text{L}$  of 0.3% DNFB (in 3:1 acetone:olive oil) on the ear once a day, for a total of 3 days. The mice were euthanized 24 hours after the last challenge. In some experiments, mice were treated with  $500\mu\text{g}$  of a Rat

IgG2a isotype monoclonal antibody (BioXcell, clone 2A3), an anti-mouse IL-17A monoclonal antibody (BioXcell, clone 17F3), or an anti-mouse IL-1R monoclonal antibody (BioXcell, clone JAMA-147), one day prior and one day after the first challenge with DNFB.

### **Preparation of dermal sheets**

The dorsal and ventral sides of the mouse ear were split mechanically and placed dermis side down in a 24 wells plate in RPMI 1640 containing 0.25 mg/mL of Liberase TL (Roche, Diagnostics Corp.) and 10 mg/mL DNase I (Sigma-Aldrich). Ears were incubated for 90 min at 37° C in a 24-well plate. Dermal cell suspensions were prepared by dissociation on 40 µm cell strainer (Falcon) in PBS containing 0.05% BSA and 20 mM EDTA.

### **Antibodies and flow cytometry**

Single cell suspensions from the ear were obtained as described above. For analysis of surface markers and intracellular cytokines, some cells were incubated for 4 h with 10 mg/mL of brefeldin A, 50 ng/mL of PMA and 500 ng/mL ionomycin (Sigma-Aldrich). Before staining, cells were incubated with anti-mouse CD16/CD32 mouse Fc block (eBioscience) and 10% rat-IgG in PBS containing 0.1% BSA. Cells were stained for dead cells with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes) and surface markers (CD4

[eBioscience, clone RM4-5], CD8b [BioLegend, clone YTS156.7.7], CD45 [eBioscience, clone 30-F11], Ly6G [eBioscience, clone 1A8-Ly6g], CD11b [eBioscience, clone M1/70]) followed by fixation with 2% of formaldehyde and permeabilization with 0.2% saponin/PBS. Intracellular cytokine staining was performed for pro-IL-1 $\beta$  (eBioscience, clone NJTEN3). The data were collected using LSRII flow cytometer (BD) and analyzed using FlowJo software (Tree Star).

### **RNA isolation, purification, and quantitative real-time PCR.**

Total RNA was extracted from ear tissue samples in 500 $\mu$ L of RLT lysis buffer (QIAGEN). The sample was homogenized using a tissue homogenizer (FastPrep-24, MP Biomedical), and total RNA was extracted according to the recommendations of the manufacturer and further purified using the RNeasy Mini kit (QIAGEN). RNA was reverse transcribed using high capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time RT-PCR was performed on a ViiA™ 7 Real-Time PCR System (Applied Biosystems). Relative quantities of mRNA for several genes were determined using SYBR Green PCR Master Mix (Applied Biosystems) and by the comparative threshold cycle method, as described by the manufacturer. mRNA levels for each sample were normalized to the ribosomal protein S11 gene (RPS11). The primer sequences were as follows: *Rps11*, forward, 5'-CGTGACGAAGATGAAGATGC-3' and reverse, 5'-GCACATTGAATCGCACAGTC-3'; *Ii17*, forward, 5'-

CATGAGTCCAGGGAGAGCTT-3' and reverse, 5'-  
GCTGAGCTTTGAGGGATGAT-3'; *Tnfa*, forward, 5'-  
TCACTGGAGCCTCGAATGTC-3' and reverse, 5'-  
GTGAGGAAGGCTGTGCATTG-3'; *Il1b*, forward, 5'-  
TTGACGGACCCCAAAGAT-3' and reverse, 5'- GATGTGCTGCTGCGAGATT-  
3'; *Cxcl1*, forward, 5'-GCACCCAAACCGAAGTCATA-3' and reverse, 5'-  
CTTGGGGACACCTTTTAGCA-3'; and *Ccl2*, forward, 5'-  
GCTTCTGGGCCTGCTGTTCA-3' and reverse, 5'-  
AGCTCTCCAGCCTACTCATT-3'.

### **Microbiota collection, sequencing, and analysis**

Microbiota samples were collected from the ear of mice using a swab (Catch-all Sample Collection Swab, Epicentre) moistened in Yeast Cell Lysis Buffer (from MasterPure Yeast DNA Purification Kit; Epicentre). DNA was isolated from swab specimens using the PureLink Genomic DNA Mini Kit (Invitrogen) and amplification of the 16S-V4 region for the murine samples, and 16S-V1-V3 region for the human samples, was performed as previously described (Hannigan et al. 2014; Meisel et al. 2016). Sequencing of 16S rRNA amplicons was performed at the Penn Next Generation Sequencing Core using the Illumina MiSeq platform with 150 bp paired-end 'V4' chemistry for murine samples and with 300 bp paired-end 'V1-V3' chemistry for the human samples. For the fecal samples, DNA was isolated using the PowerSoil DNA Isolation Kit (Mo Bio) and

sequencing of the 16S rRNA amplicons was conducted using 250bp paired-end 'V4' chemistry with dual index primers (Kozich et al. 2013).

## **QUANTIFICATION AND STATISTICAL ANALYSIS**

### **Pre-processing and community characterization of 16S rRNA sequence data**

Sequence pre-processing followed methods previously described (Hannigan et al. 2014), but modified by subsampling at 5000 sequences per sample for murine samples, and at 1000 sequences per sample for human samples. QIIME 1.8.0 (Caporaso et al. 2010) was used for initial stages of sequence analysis. Sequences were clustered into OTUs (operational taxonomic units, a proxy for 'species') using UCLUST (Edgar 2010) at 97% sequence similarity. Bacterial diversity was calculated using the following alpha diversity indices: Shannon diversity index and the number of observed OTUs. Relative abundance of bacteria was calculated based on taxonomic classification of sequences using the RDP classifier (Wang et al. 2007) at a confidence threshold of 0.8. Microbiota data was analyzed with the R statistical software environment ([www.r-project.org](http://www.r-project.org)). Statistical significance was determined using two-sample Wilcoxon tests and corrected for multiple comparisons by FDR where appropriate. Dirichlet

multinomial mixture modeling was performed using the R package Dirichlet Multinomial and calculated as previously reported (Loesche et al. 2016).

### **Statistical analysis**

Results represent means  $\pm$  SEM. Data were analyzed using Prism 7.0 (GraphPad Software, San Diego, CA). Statistical significance was determined by one-way ANOVA when comparing more than two groups and by an unpaired two-tailed Student's t test to compare means of lesion sizes, parasite burdens, and cytokine production from different groups of mice. Variances were equal between experimental groups. Statistically significant differences were defined as \* when  $p$  values were  $<0.05$ .

### 3.4 Results

#### Characterization of microbiota colonizing human leishmaniasis lesions and skin

Dysbiosis in skin microbiota is often associated with inflammation and disease (Grice et al. 2010; Kobayashi et al. 2015; Kong et al. 2012; Oh et al. 2013), suggesting that cutaneous lesions in leishmaniasis might also exhibit changes in the skin-residing bacterial communities. To test this, we analyzed the microbiota of 44 patients infected with *L. braziliensis* (72.7% male, 27.3% female, median age, 27 years old), with lesions present at various body sites (Table 1). We collected 2-3 skin swabs for each patient including the lesion, adjacent skin near the lesion, and unaffected contralateral skin of the same body site as the lesion (Figure 8A). Bacterial diversity was significantly lower in lesions compared to unaffected contralateral skin and adjacent skin sites, as measured by the observed species-level operational taxonomic units (OTUs) and Shannon Diversity indices (Figure 8B).

Interestingly, the skin microbiota on the adjacent skin sites appeared more similar in composition to the lesions than to the contralateral skin (Figure 9a). To quantify the similarity between each site where specimens were collected, we used the Bray Curtis dissimilarity metric of shared microbial community structure. We observed that lesion and adjacent skin shared greater microbial community structure compared to contralateral and adjacent skin (Figure 8C). This data

suggests that microbiota colonizing the lesion is shared with adjacent skin sites, which may have implications in the immune responses at those sites.

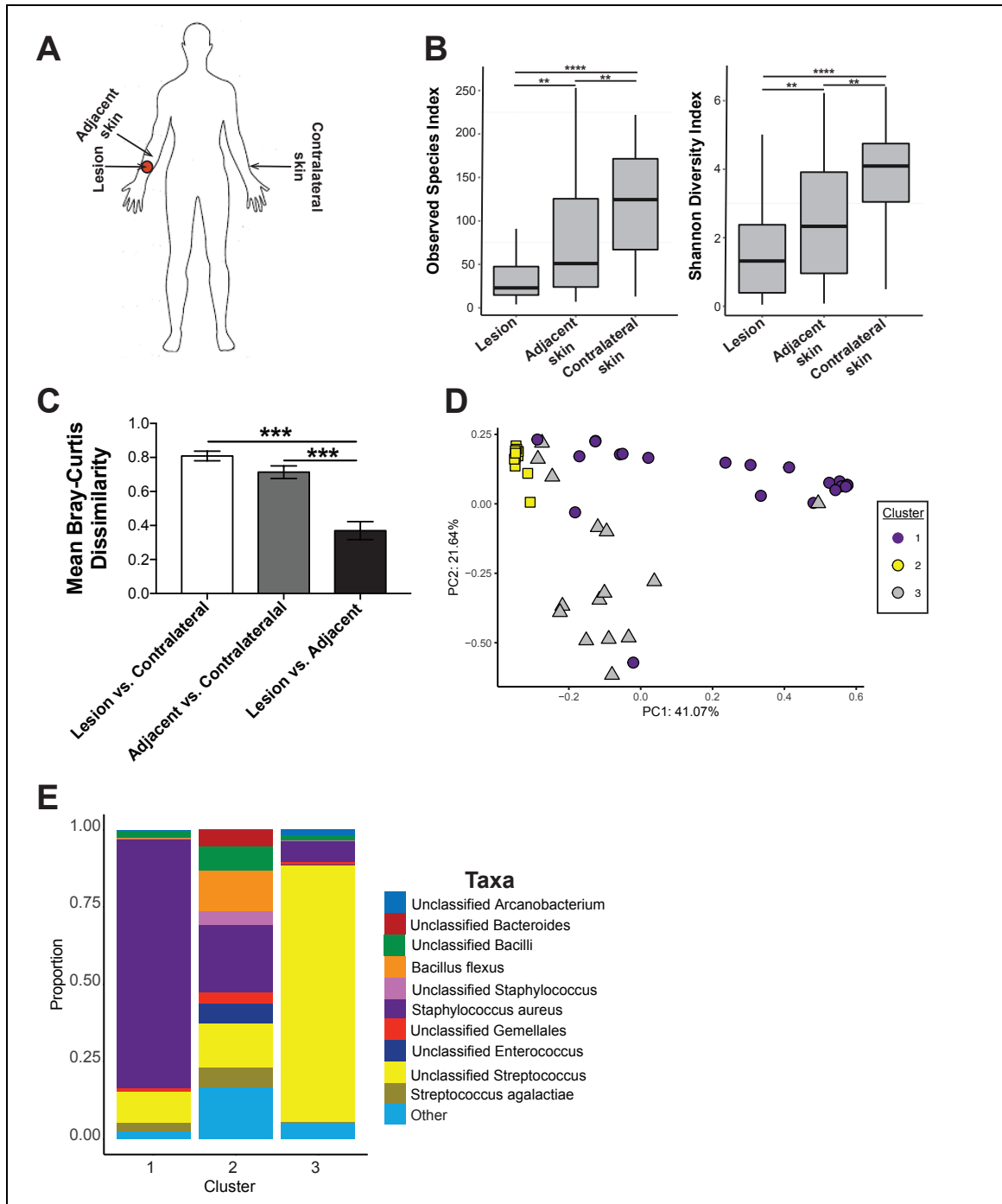
We then applied a Dirichlet multinomial mixture model-based approach to assign the lesions to different community types (CTs) based on their taxonomic composition. Lesions clustered into 3 CTs (Figure 8D and Figure 9B) with distinct bacterial compositions. The top discriminating taxa in CT1 was *Staphylococcus aureus*, CT2 displayed a heterogeneous composition with no dominating taxa, and CT3 was dominated by an unclassified species of *Streptococcus* (Figure 8E and Table 2). These results suggest that cutaneous leishmaniasis lesions are colonized with microbiota similar to other cutaneous ulcers (Kong et al. 2012; Oh et al. 2013; Loesche et al. 2016), but display less heterogeneity of the colonizing microbiota, which is driven primarily by proportions of *Staphylococcus aureus* and *Streptococcus* spp. in this cohort. Interestingly, neither bacteria were associated with larger lesion sizes (Figure 9C), but lesion size may not be a good predictor of disease severity or outcome. Additional epidemiologic studies may be needed to further evaluate the influence of the skin microbiota in cutaneous leishmaniasis, yet these results clearly demonstrate that infection with leishmania alters the skin microbiota, creating several types of dysbiosis.



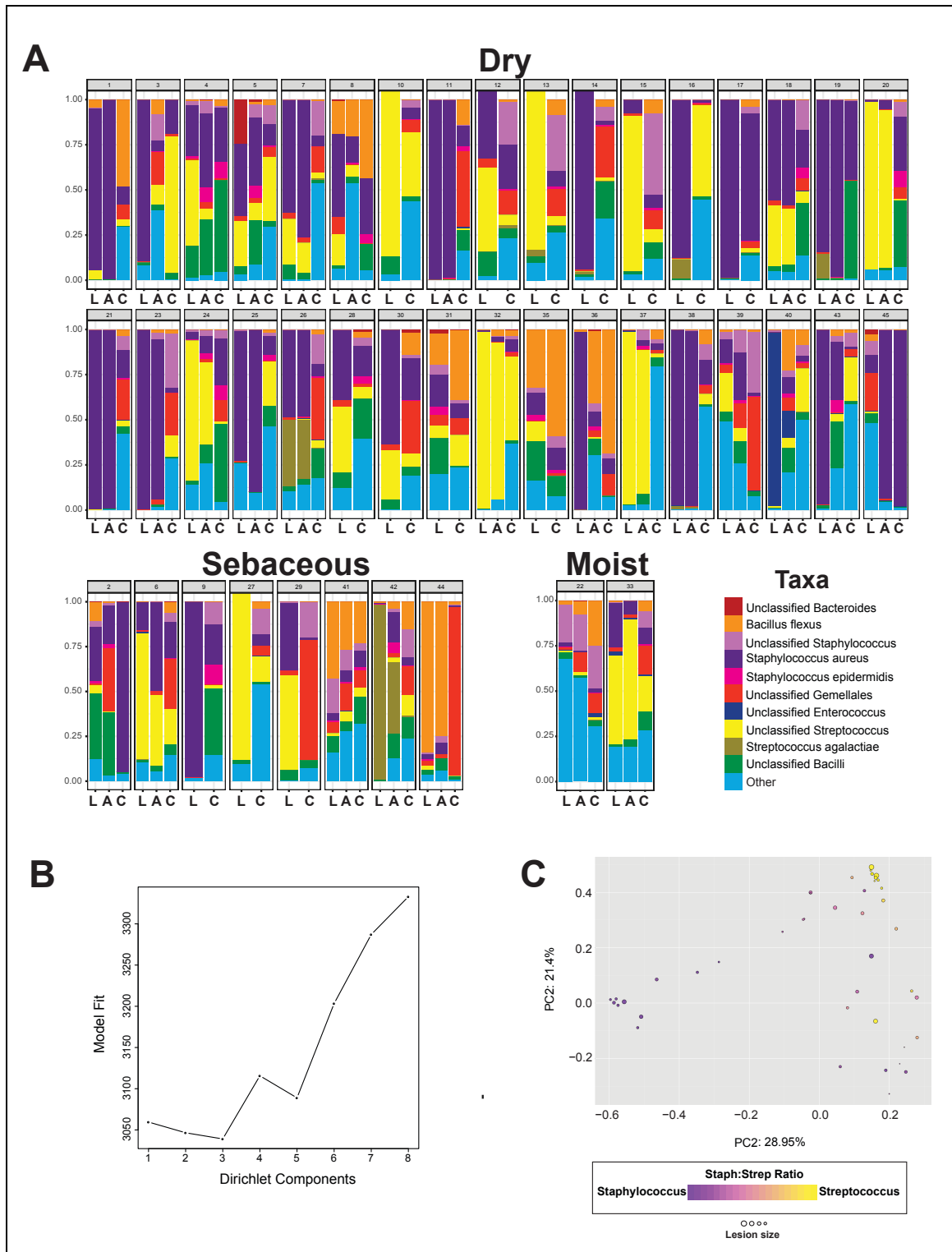
Subject ID	Sex	Age	Body Site	Lesion Size (mm2)	Duration of Lesion (Days)	Skin Test (mm2)
1	Male	22	Leg	396	30	440
2	Male	43	Neck	216	30	225
3	Male	18	Ankle	NA	40	300
4	Male	24	Leg	225	40	144
5	Male	20	Leg	660	90	725
6	Male	16	Back	500	30	440
7	Female	30	Leg	544	21	225
8	Male	36	Thigh	437	30	Negative
9	Male	31	Face	840	60	300
10	Male	22	Arm	49	30	400
11	Female	24	Thigh	25	30	210
12	Female	39	Leg	70	30	130
13	Male	22	Leg	3300	40	110
14	Female	45	Leg	180	30	378
15	Male	26	Leg	90	10	260
16	Male	21	Arm	200	60	400
17	Female	33	Leg	380	40	NA
18	Male	21	Leg	780	21	228
19	Female	20	Leg	80	30	100
20	Male	29	Leg	500	60	180
21	Female	35	Leg	100	60	255
22	Male	37	Foot	24	60	300
23	Female	26	Arm	130	30	285
24	Male	25	Leg	150	21	180
25	Male	50	Leg	270	30	272
26	Male	55	NA	1575	60	1085
27	Male	18	Head	192	34	130
28	Male	19	Leg	480	14	208
29	Female	24	Abdomen	325	NA	700
30	Male	40	Leg	130	20	460
31	Male	57	Leg	35	45	132
32	Male	18	Leg	49	20	400
33	Male	19	Foot	306	15	441
34	Male	28	Leg	25	15	49
35	Male	39	Leg	77	30	255
36	Male	31	Leg	330	90	130
37	Female	24	Leg	1476	60	625
38	Male	63	Leg	272	20	196
39	Male	20	Arm	1377	45	225
40	Female	16	Chest	30	20	156
41	Female	24	Back	255	30	144
42	Female	64	Leg	216	30	NA
43	Male	33	Abdomen	207	40	289
44	Male	59	Thigh	340	90	255

**Table 1: Information about samples collected from cutaneous leishmaniasis patients.**

Swabs were collected from these cutaneous leishmaniasis patients prior to treatment. All cutaneous leishmaniasis patients were seen at the health post in Corte de Pedra, Bahia, Brazil, which is a well-known area of *L. braziliensis* transmission. The criteria for diagnosis were a clinical picture characteristic of cutaneous leishmaniasis in conjunction with parasite isolation or a positive delayed-type hypersensitivity response to *Leishmania* antigen, plus histological features of cutaneous leishmaniasis.



**Figure 8: Lesions from cutaneous leishmaniasis patients also have a dysbiotic skin microbiota.** (A) Swabs were collected from the lesion, nearby adjacent skin, and contralateral skin sites for 16S rRNA analysis. (B) Bacterial diversity was assessed by the number of observed species-level OTUs and Shannon Index. (C) Bar charts represent intragroup mean Bray-Curtis dissimilarity between each skin site. (D) PCoA values for weighted UniFrac analysis were plotted and colored based on the Dirichlet multinomial cluster assignment. (E) Stacked bar charts represent the proportion of the top 10 taxa present in each Dirichlet cluster. Samples were collected from an n = 44 patients.



**Figure 9: Samples from all patients are diverse and Dirichlet multinomial clusters lesions into 3 community types. (A)** Stacked bar charts represent the proportion of the top 10 taxa present in each sample. Patients are identified by number and skin type is identified as lesion (L),

adjacent skin sites (A), or contralateral skin sites (C). **(B)** Laplace approximation was used to measure the model fit of the Dirichlet multinomial mixture analysis. The lowest value (3) indicates the best fit for the model. **(C)** PCoA values for weighted UniFrac analysis were plotted and colored based on the ratio of the abundances of *Staphylococcus* spp. to *Streptococcus* spp. in each lesions sample and circle size is based on size of the lesion of each sample.

Taxa	Cluster 1	Cluster 2	Cluster 3
<b>Staphylococcus aureus</b>	0.788992731	0.16077901	0.049802691
<b>Unclassified Streptococcus</b>	0.022511662	0.11087683	0.814618671
<b>Unclassified Bacilli</b>	0.04901982	0.10691883	0.018451401
<b>Unclassified Gemellales</b>	0.030965307	0.06667456	0.013448359
<b>Unclassified Staphylococcus</b>	0.013819064	0.06478127	0.005975455
<b>Bacillus flexus</b>	0.002613152	0.06594624	0.00479366
<b>Unclassified Staphylococcus</b>	0.010710605	0.02253503	0.004936899
<b>Unclassified Streptococcus</b>	0.007199348	0.0130796	0.01322084
<b>Unclassified Bacillales</b>	0.002463076	0.02473744	0.001617316
<b>Staphylococcus epidermidis</b>	0.001303855	0.02767135	0.001574108

**Table 2: Top 10 discriminating taxa that make up the Dirichlet multinomial clusters.**

Dirichlet multinomial mixture model-based approach was used to assign the lesions into different clusters based on their taxonomic composition. This tables provides a list of the top 10 discriminating taxa that make up each cluster and their proportional contribution to the cluster.

### ***L. major* infection induces changes to the skin microbiota in mouse models**

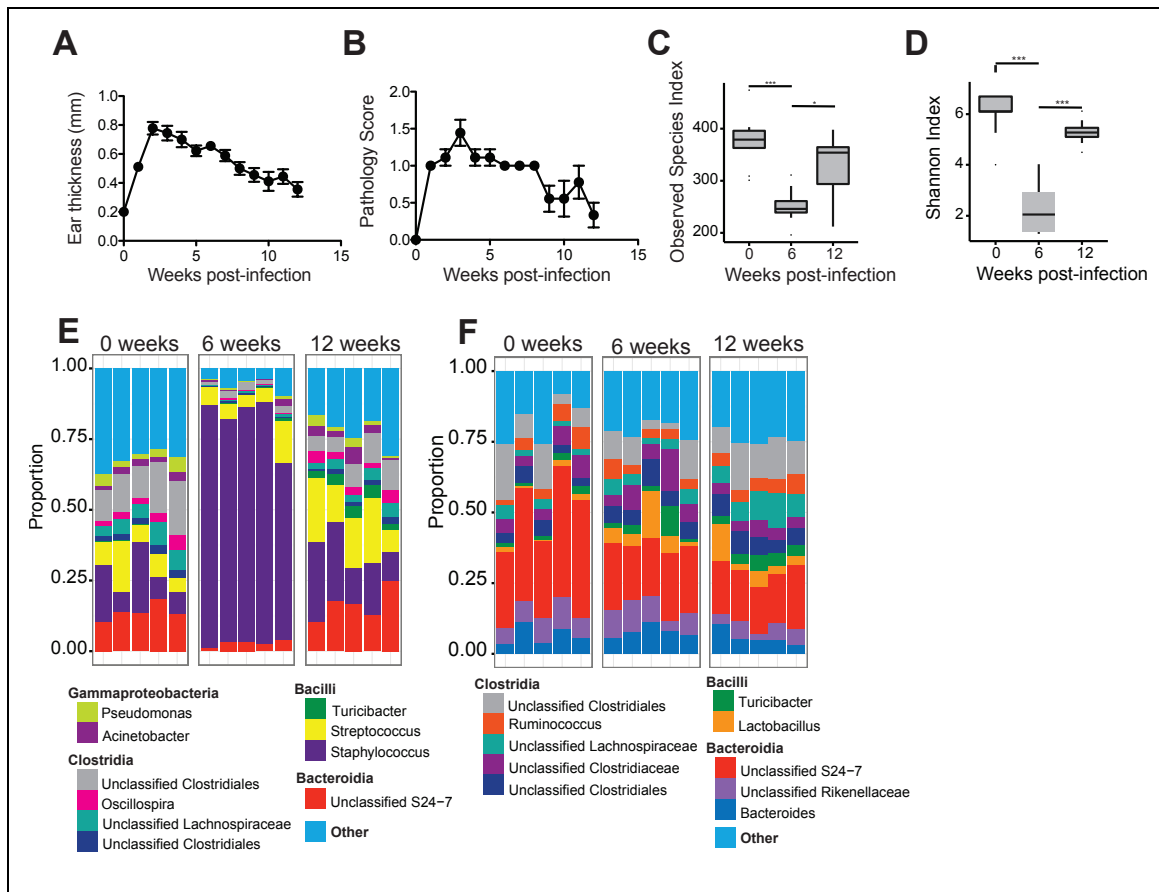
Since the influence on disease of a dysbiosis is difficult to evaluate in humans, we employed a mouse model of leishmaniasis to assess the role dysbiosis might play in cutaneous leishmaniasis. C57BL/6 mice were infected in the ear with *L. major* parasites, which led to the development of a lesion that resolved by 12 weeks post-infection (Figure 10A-B). Prior to infection, and at 6 and 12 weeks post-infection, swabs were collected from the ventral and dorsal ear skin and sequencing of the 16S ribosomal RNA gene was employed to assess skin microbial diversity and composition. Alpha diversity, as measured by

the number of observed species-level OTUs and Shannon Diversity indices, decreased at 6 weeks post-infection, but returned to pre-infection levels upon lesion resolution (Figure 10C-D). This shift in alpha diversity was paralleled by a significant increase in the relative abundance of *Staphylococcus* spp. after lesion development that returned to pre-infection levels once the lesions resolved (Figure 10E). MALDI-Tof mass spectrometry identified the *Staphylococcus* species associated with *L. major* infection as *S. xylosus* (data not shown), a common commensal bacteria found on mouse skin (Nagase et al. 2002). Since infections can often lead to changes in the intestinal microbiota (Kamdar et al. 2016; Lozupone et al. 2013), we also analyzed the fecal microbiota of infected mice, but found no significant changes in the fecal bacterial populations throughout the course of infection with *L. major* (Figure 10F), demonstrating that dysbiosis caused by infection is localized to the skin.

### ***L. major* induced dysbiosis differs depending on the severity of the disease**

Inflammatory responses induced by a variety of skin insults lead to changes in the skin microbiota (Grice et al. 2010; Gontcharova et al. 2010; Kong et al. 2012; Oh et al. 2013; Loesche et al. 2016), but whether the magnitude of the insult alters the nature or degree of the dysbiosis is not known. To address this we compared the microbiota from *L. major* infected C57BL/6 mice that resolve their infection and BALB/c mice that develop severely ulcerated non-healing lesions (Figure 11A-B) (Scott and Novais 2016). Similar to C57BL/6

mice, BALB/c mice had significantly lower alpha diversity at 6 weeks post-infection (Figure 11C). However, in contrast to the dominance of *Staphylococcus* spp. found on lesions of C57BL/6 mice, BALB/c mice had a dominance of *Streptococcus* spp. at 6 weeks post-infection, (Figure 11D). To rule out the possibility that the increase in *Streptococcus* in non-healing BALB/c mice was due to differences in the mouse strain, we depleted IL-12 in C57BL/6 mice, which

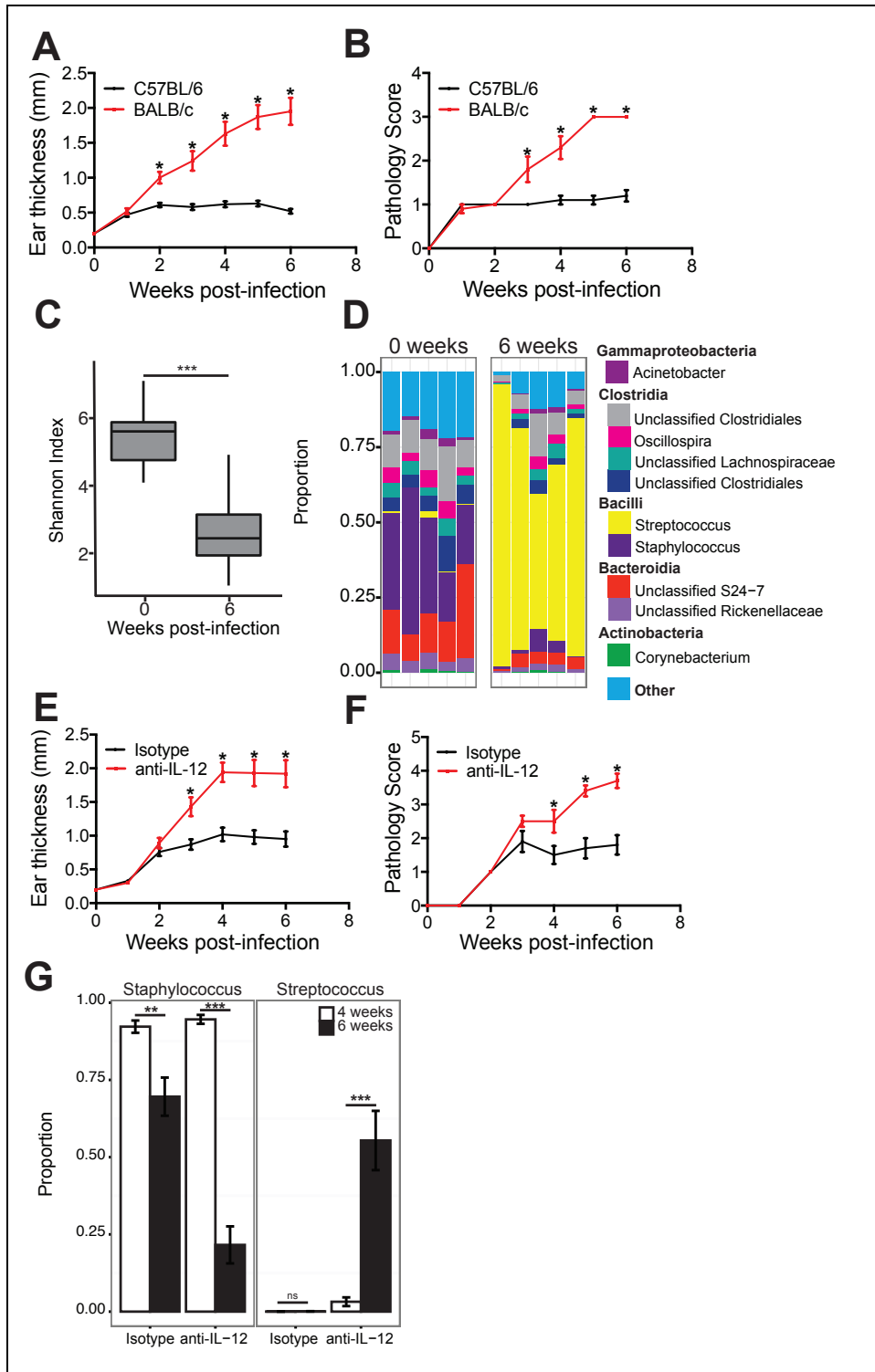


**Figure 10: *L. major* infection alters the skin microbiota.** C57BL/6 mice were intradermally infected in the ear with  $2 \times 10^6$  *L. major* parasites. **(A)** Lesion size and **(B)** pathology were assessed over 12 weeks of infection. Swabs were collected from the ear at 0, 6, and 12 weeks post-infection and bacterial diversity was assessed by **(C)** number of observed species-level OTUs and **(D)** Shannon Index. Stacked bar charts represent the proportion of the top 10 taxa present **(E)** from ear swabs and **(F)** from fecal pellets at 0, 6, and 12 weeks post-infection. Each column represents the proportion of taxa for an individual mouse. Data represent two independent experiments (For skin swabs,  $n = 15$  mice and for fecal pellets,  $n = 10$  mice).

leads to non-healing lesions similar to those seen in BALB/c mice(Heinzel et al. 1989; Scharton-Kersten et al. 1995). As expected, anti-IL-12 mAb treated mice developed large non-healing lesions (Figure 11E-F). At 4 weeks post-infection *Staphylococcus* spp. made up a high proportion of the skin microbiota in both groups of mice (Figure 11G). However, while the relative abundance of *Streptococcus* spp. remained less than 1% of the total population in control mice, it increased significantly in anti-IL-12 treated mice to >50% relative abundance (Figure 11G), further demonstrating that *Streptococcus* spp. are associated with more severely ulcerated lesions. Taken together, our data suggest that *L. major* infection elicits severity-dependent changes in the skin microbiota.

### ***S. xylosus* mediated inflammation is dependent on skin barrier integrity**

To determine if the dysbiosis caused by *L. major* infection would influence skin inflammatory responses, we topically associated naïve mice with *S. xylosus* (Figure 12A). One week following colonization with *S. xylosus* mice exhibited a significantly higher relative and absolute abundance of *Staphylococcus* spp. compared with naïve mice by culture-independent (Figure 12B) and culture-dependent assays (Figure 12C). CD4+ T cells, CD8+ T cells, CD11b+ myeloid cells (Figure 12D), and cytokine production (data not shown) were unchanged in skin colonized with *S. xylosus* compared to naïve skin. To determine if *S. xylosus* incites inflammation upon breach of the skin barrier, we injected mice intradermally with *S. xylosus* and analyzed the inflammatory response in the skin.



**Figure 11: Skin microbiota alterations in *L. major* infection are dependent on disease severity.** C57BL/6 and BALB/c mice were intradermally infected with *L. major* parasites. Lesional severity was assessed by (A) ear thickness and (B) a pathology score over the course of



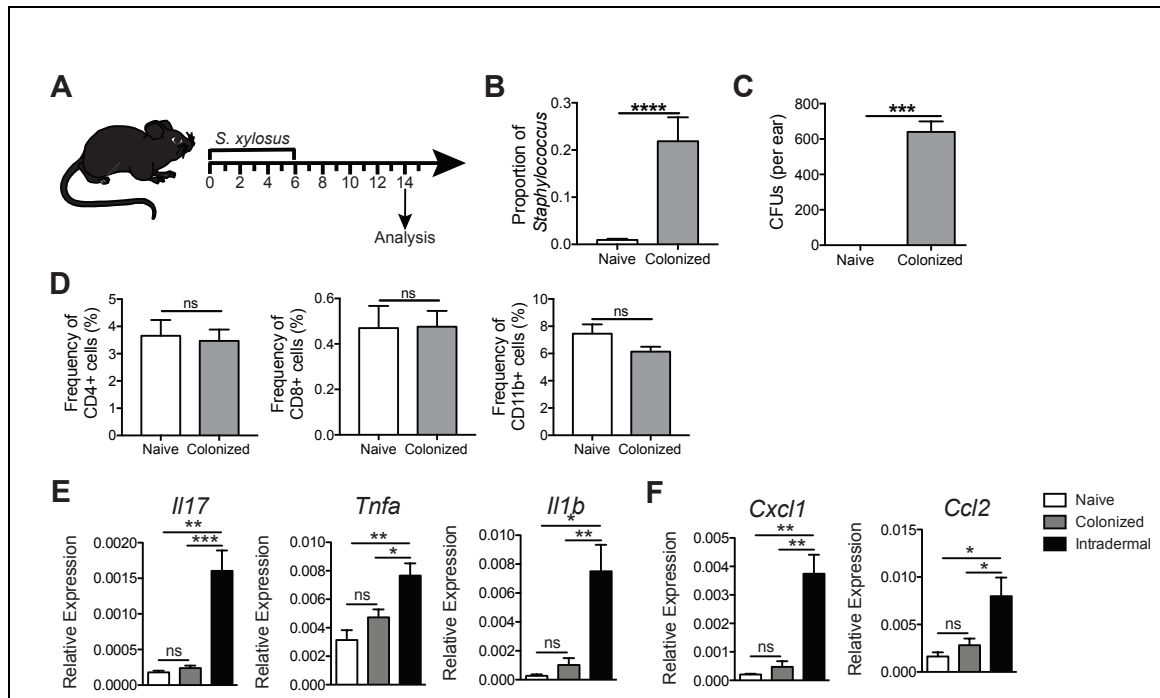
infection. Swabs for sequencing of 16S rRNA genes were collected from the lesions at 0 and 6 weeks post-infection. **(C)** Alpha diversity was assessed by Shannon Index. **(D)** Stacked bar charts represent the proportion of the top 10 taxa present in each sample. Data are representative of two independent experiments (n=5-10 mice per group). C57BL/6 mice were treated with an isotype or anti-IL-12 mAb and intradermally infected in the ear with *L. major* parasites. Lesional severity was assessed by **(E)** ear thickness and **(F)** a pathology score over the course of infection. Anti-IL-12 mAb treated mice were euthanized at 6 weeks post-infection due to severe disease. **(G)** Swabs were collected from the lesions at 4 and 6 weeks post-infection and proportions of *Staphylococcus* and *Streptococcus* were assessed. Data are representative of two independent experiments (n=10 mice/group).

These mice had significantly higher expression of *Il17*, *Tnfa*, *Il1b*, *Cxcl1*, and *Ccl2* compared with either naïve or colonized mice (Figure 12E-F), suggesting that *S. xylosus* might contribute to skin inflammation when the skin barrier is compromised.

While skin colonized with *S. xylosus* appeared immunologically normal, based on the results above we hypothesized that the response to damage might differ between normal and dysbiotic skin. We tested this idea using a model of contact hypersensitivity in which sensitizing and challenging the skin with a known skin irritant, dinitrofluorobenzene (DNFB), increases transepidermal water loss, an indication of skin barrier dysfunction (Figure 13A). Naïve C57BL/6 mice were colonized with *S. xylosus* prior to sensitization with DNFB (Figure 13B). DNFB challenge resulted in a significant increase in neutrophils (CD11b+ Ly6G+) and expression of pro-IL-1 $\beta$  from myeloid cells (Figure 13C-D). Since IL-17 and IL-1 can both lead to an increase in neutrophil recruitment, we investigated whether these cytokines played a role in the increase of neutrophils in *S. xylosus* treated mice. Mice colonized with *S. xylosus* were treated with an isotype control mAb, anti-IL-17A mAb, or anti-IL-1R mAb prior to DNFB challenge (Figure 13E),

and neutralizing IL-17 or IL-1 decreased neutrophil recruitment (Figure 13F).

Thus, it appears that once the integrity of the skin is compromised a commensal such as *S. xylosus* can induce IL-17 and IL-1 expression, leading to increased inflammation.



**Figure 12: *Staphylococcus xylosus* isolated from *L. major* lesions causes inflammation only when injected intradermally.** (A) C57BL/6 mice were topically colonized with  $10^8$ - $10^9$  *S. xylosus* every other day for a total of 4 applications; naïve mice were unassociated. (B) Prior to and 14 days post colonization, swabs were collected to analyze the proportion of *Staphylococcus*. (C) Ear lysates from naïve and *S. xylosus* colonized mice were cultured on mannitol salt agar plates and colony forming units were counted after overnight incubation at 37°C. (D) Flow cytometry analysis was performed for CD4+, CD8+, and CD11b+ cells in the ears of naïve or colonized mice 14 days post-association. Cells were pregated on live, singlet, CD45+ cells. Data are representative of two independent experiments (n = 4 mice/group). C57BL/6 mice were topically colonized or intradermally infected in the ear with *S. xylosus*. Fourteen days later, skin was harvested and mRNA expression was assessed for (E) cytokine and (F) chemokine genes. Data are representative of one experiment (n = 5 mice/group).

To determine if colonization with *Streptococcus* spp. might have a similar effect, we isolated *Streptococcus* from *L. major* infected mice that had been

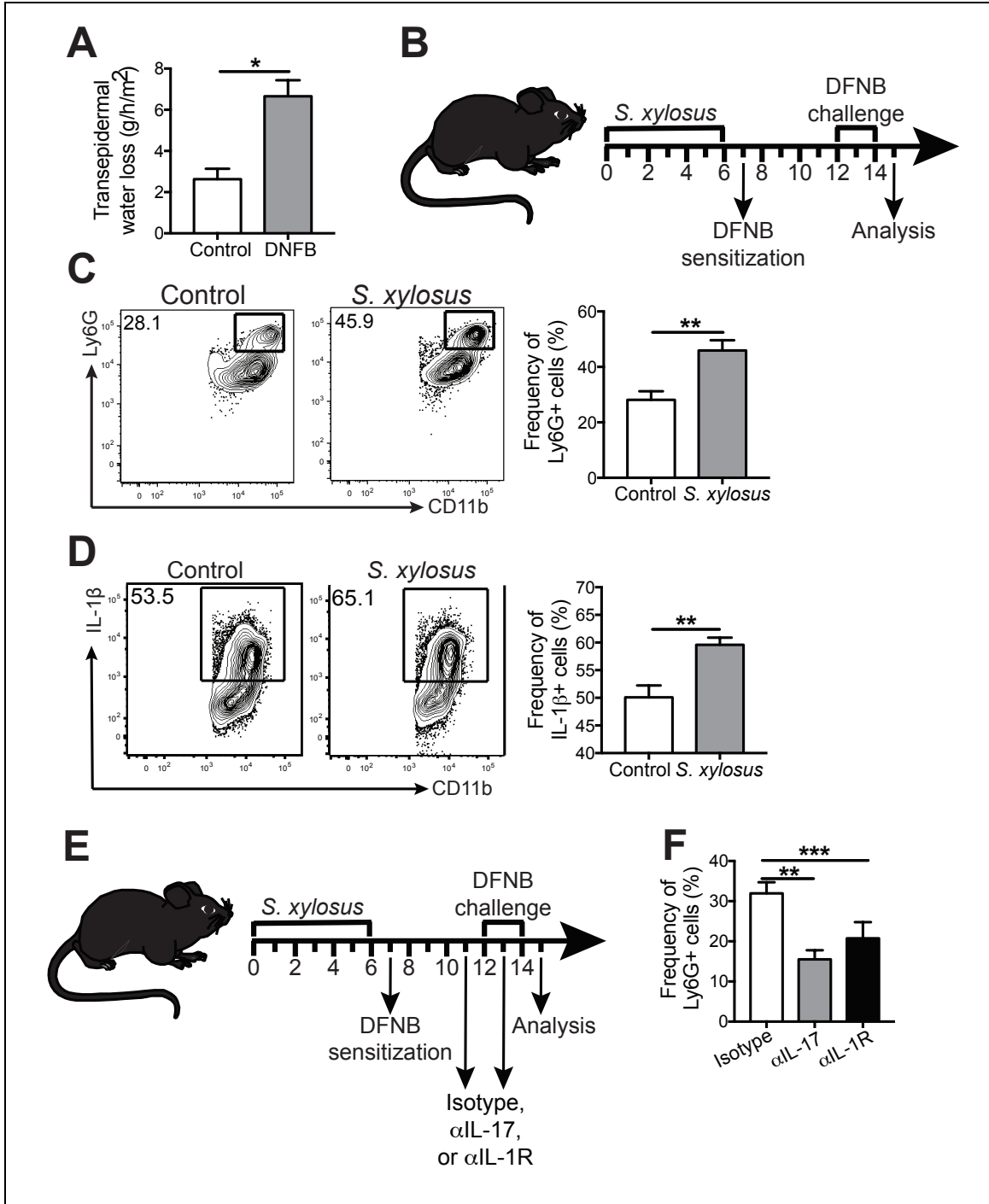
treated with anti-IL-12 mAb. The immune responses in mice sensitized and challenged with DNFB colonized with *Streptococcus* was unchanged (Figure 14A). However, we were unable to achieve stable colonization with the streptococcal isolate (Figure 14B), suggesting that this particular lesion-associated *Streptococcus* isolate requires additional as yet undefined nutrients or other conditions to colonize normal skin.

### ***L. major*-induced dysbiosis is transmissible to uninfected skin**

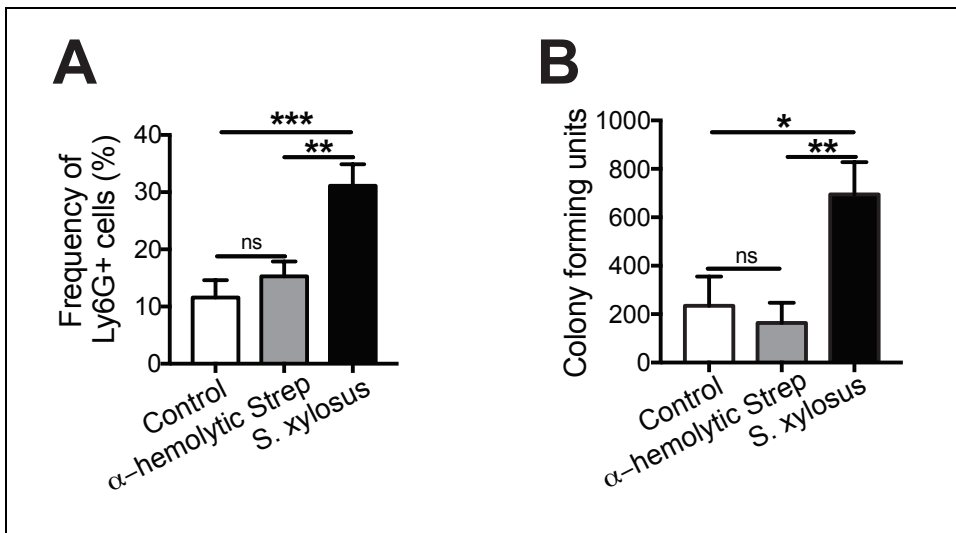
The observation that the lesional microbiota of human cutaneous leishmaniasis extends to adjacent seemingly normal skin sites prompted us to ask if the same was true in the mouse model of *L. major* infection. To answer this question, we compared the bacterial composition at the lesion site (infected ear) and the contralateral ear of infected mice. As expected, the infected ear was dominated by *Staphylococcus* spp. at the peak of infection. Interestingly, the contralateral ear also had a high proportion of *Staphylococcus* spp., despite the absence of infection (Figure 15A). We also observed higher bacterial loads on the infected and contralateral ears when compared to naïve skin (Figure 15B). These data demonstrate that in the mouse model, the dysbiotic microbiota caused by *L. major* infection is transmissible to the non-inflamed, contralateral ear.

A dysbiotic intestinal microbiota is often transmissible by simply co-housing mice (Elinav et al. 2011a; Zenewicz et al. 2013). Whether transmission of the skin microbiota also occurs is less clear, although co-habiting families may

share their skin microbiota (Song et al. 2013). To directly address this issue we tested if naïve mice co-housed with *L. major* infected mice might acquire their dysbiotic microbiota. C57BL/6 mice were infected with *L. major* and co-housed



**Figure 13: *S. xylosus* colonization exacerbates skin inflammation during contact hypersensitivity.** (A) C57BL/6 mice were sensitized with DNFB or vehicle control on the belly and challenged with DNFB or vehicle 5 days later. Transepidermal water loss was measured on ear skin of vehicle control and DNFB treated mice. (B) C57BL/6 mice were topically associated with  $10^8$ - $10^9$  *S. xylosus* every other day for a total of 4 applications and control C57BL/6 mice were left unassociated. The next day, control and *S. xylosus* associated mice were sensitized on the belly with DNFB. 5 days later, control and *S. xylosus* associated mice were challenged with DNFB. Representative flow cytometry plots and graphs depict the expression of (C) CD11b+ Ly6G+ cells and (D) CD11b+ IL-1 $\beta$ + cells. (E) C57BL/6 mice were topically associated with  $10^8$ - $10^9$  *S. xylosus* every other day for a total of 4 applications and then treated with isotype, anti-IL-17, or anti-IL-1R mAbs prior to sensitization and challenge with DNFB. (F) Graphs depict the expression of CD11b+ Ly6G+ cells in the skin of treated mice. All data are representative of two independent experiments (n = 5 mice/group).



**Figure 14. *Streptococcus* does not colonize naïve skin and fails to exacerbate skin inflammation during contact hypersensitivity.** (A) C57BL/6 mice were topically associated with  $10^8$ - $10^9$  of an alpha hemolytic streptococcal isolate or *S. xylosus* every other day for 4 applications and control C57BL/6 mice were left unassociated. The next day, all mice were treated on the belly with DNFB. Five days later, control alpha hemolytic *Streptococcus* and *S. xylosus* mice were challenged with DNFB. Bar graphs of skin cells depict the abundance of CD11b+ Ly6G+ cells present in the ear. (B) Colony forming units were measured after skin homogenates were cultured on tryptic soy blood agar plates overnight from the ears of control and alpha hemolytic or *S. xylosus* associated mice. Data are representative of two independent experiments (n = 5 mice/group).

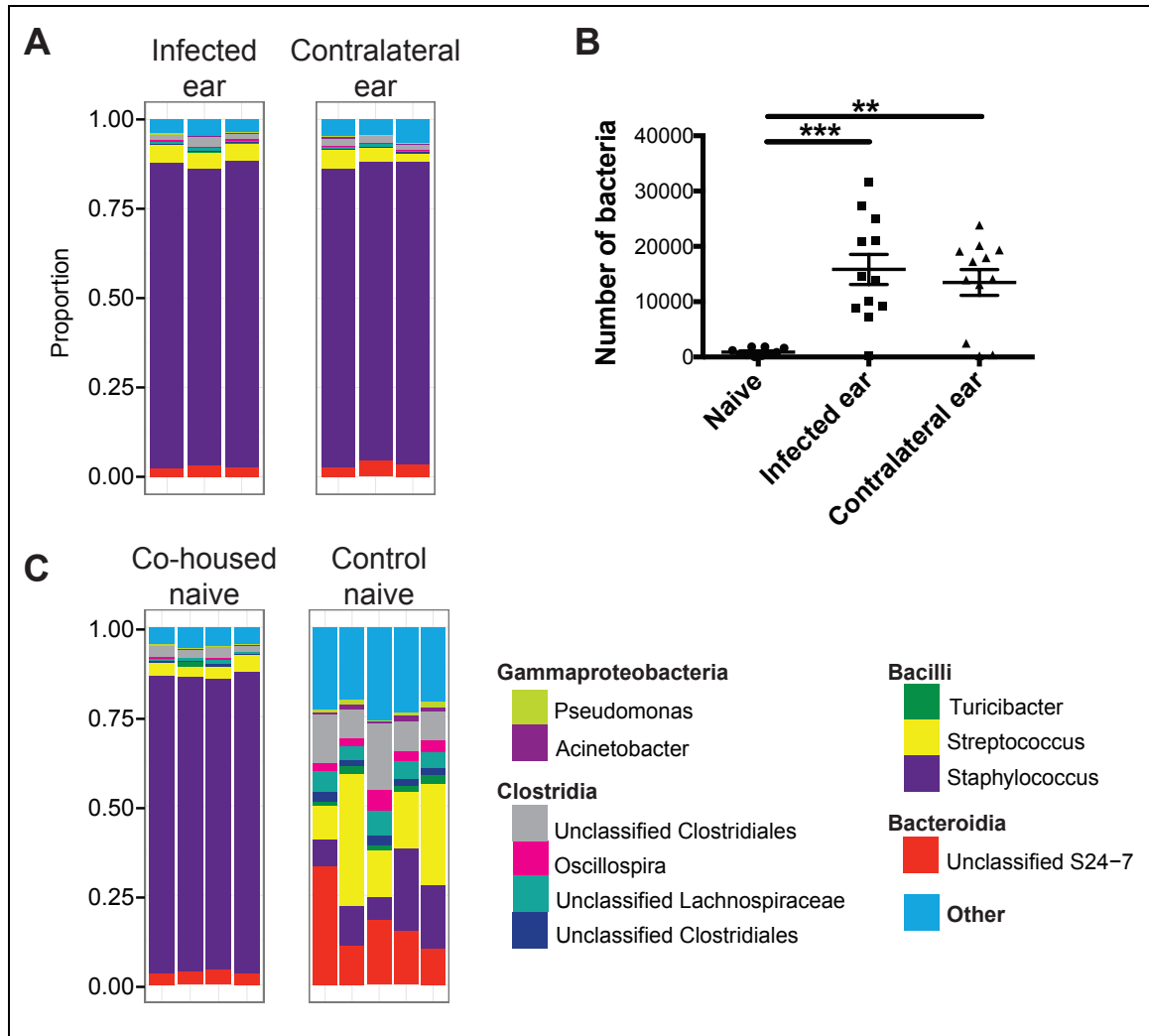
with naïve mice for 6 weeks, while a group of control naïve mice were housed separately. Similar to the infected and contralateral ears, the skin of the co-

housed naïve mice also acquired a high abundance of *Staphylococcus* spp., while the control naïve mice maintained a diverse population of bacteria (Figure 15C). Our data demonstrate that the dysbiotic skin microbiota caused by *L. major* infection is transmissible to naïve mice and allows us to assess the consequences of this acquisition in inflammatory responses occurring in the skin.

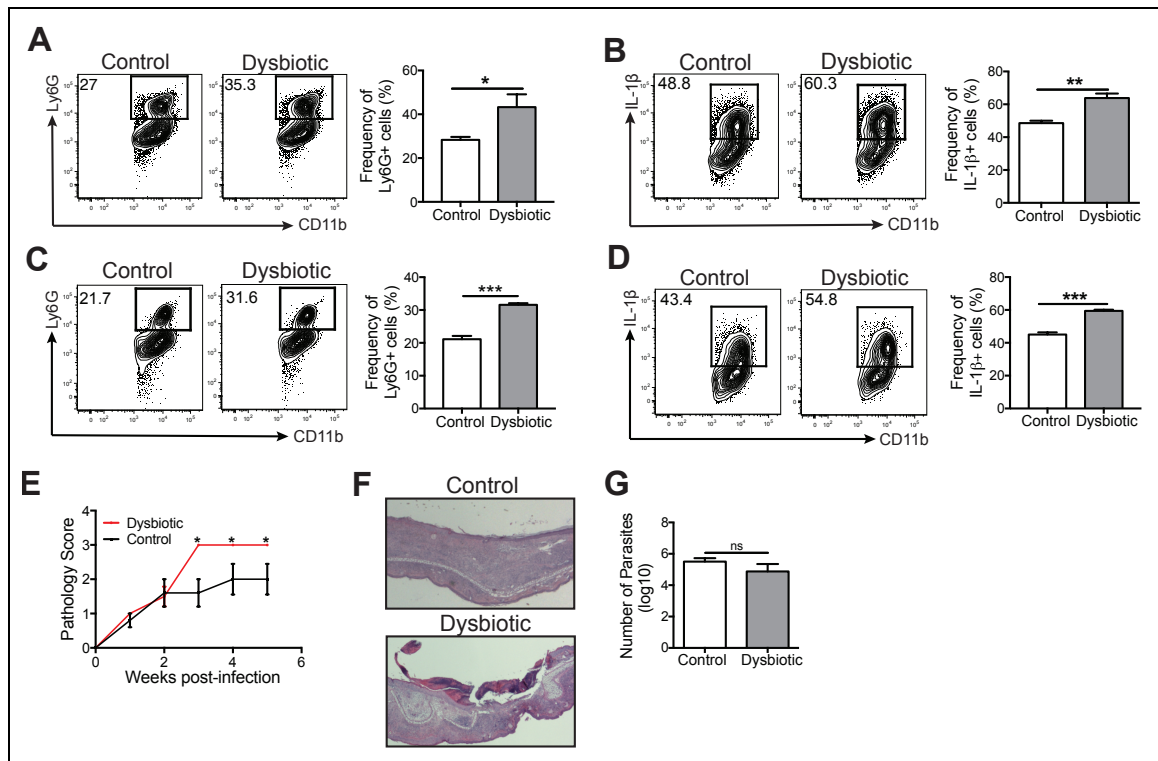
### ***L. major*-induced dysbiosis exacerbates disease during inflammation and infection**

While we and others have shown that colonizing mice with a single organism at high levels can alter immune responses (Figure 13) (Naik et al. 2012; Naik et al. 2015), whether a naturally transmitted dysbiosis would alter immune skin immune responses has not been tested. To assess this, we co-housed naïve mice with *L. major* infected mice for 6 weeks to create “naïve” dysbiotic mice. Control mice were housed separately and never exposed to *L. major* infected mice. We then compared the contact hypersensitivity responses of both groups of mice to DNFB. Dysbiotic co-housed mice had significantly more neutrophils and pro-IL-1 $\beta$  production in the skin than control mice (Figure 16A-B), similar to mice colonized with high numbers of bacteria.

Taken together, our results suggested that mice with dysbiotic skin might respond differently to infection with *L. major* when compared with normal mice. To determine if this was the case, naïve mice were co-housed with *L. major* infected mice for 6 weeks and then infected with *L. major*. At 5 weeks post-infection, we analyzed the inflammatory cells and cytokines in the lesions of



**Figure 15. *L. major* induced dysbiosis is transmissible to uninfected skin. (A)** C57BL/6 mice were intradermally infected with *L. major* and swabs were collected from the infected and contralateral ears at 6 weeks post-infected for 16S rRNA gene analysis. Stacked bar charts represent the proportion of the top 10 taxa present in each sample. Data are representative of three independent experiments (n=5-10 mice per group). **(B)** Swabs from naïve or *L. major* infected C57BL/6 mice were cultured on mannitol salt agar plates and CFUs were counted to determine bacteria burden. Data are representative of 1 experiment (For naïve mice, n = 10; for infected and contralateral ears, n = 12). **(C)** Naïve C57BL/6 mice were co-housed with *L. major* infected mice for 6 weeks, while control naïve mice were housed separately. Swabs were collected from co-housed naïve and control naïve mice. Stacked bar charts represent the proportion of taxa present in each sample. Data are representative of two independent experiments (For infected mice, n = 15 mice; for co-housed naïve, n = 10 mice; for control naïve, n = 5 mice).



**Figure 16: Dysbiosis exacerbates inflammation during DNFB treatment and *L. major* infection.** Naïve C57BL/6 mice acquired dysbiotic microbiota after co-housing with *L. major* infected mice for 6 weeks. Control and dysbiotic mice were then sensitized and challenged with DNFB. Representative flow cytometry plots and graphs of skin cells depict the expression of (A) CD11b+ Ly6G+ cells and (B) CD11b+ IL-1β+ cells. Control and dysbiotic mice were intradermally infected with *L. major* parasites and the cells from the lesions were collected at 5 weeks post-infection. Representative flow cytometry plots and graphs of skin cells depict the expression of (C) CD11b+ Ly6G+ cells and (D) CD11b+ IL-1β+ cells. (E) A pathology score was used to assess disease severity over 5 weeks post-infection. (F) Representative ear skin sections stained with hematoxylin and eosin of *L. major* infected control and dysbiotic mice. (G) Parasite burdens were assessed using a limiting dilution assay after 5 weeks post-infection. Data are representative of two independent experiments (For dysbiotic mice, n = 4 mice; for control mice, n = 5).

control and dysbiotic mice. Similar to DNFB challenge, *L. major* infected skin had significantly more neutrophils and IL-1β in dysbiotic mice compared to control mice (Figure 16C-D). Furthermore, the dysbiotic mice had significantly greater lesion severity, characterized by increased skin ulceration, than control mice (Figure 16E-F) despite similar parasite burdens (Figure 16G). These findings



demonstrate that the skin microbiota influences the magnitude of lesion severity following infection with *L. major*.

### 3.5 Discussion

Interactions between the immune system and the microbiota can be either beneficial or harmful, depending on the context (Gaboriau-Routhiau et al. 2009; Naik et al. 2012; Atarashi et al. 2013; Naik et al. 2015; Kobayashi et al. 2015). In our studies, we found that leishmania infections in humans and mice change the composition of the skin microbiota. The nature of the changes in mice differed depending on the severity of inflammation, with *Staphylococcus* spp. dominant in moderate lesions and *Streptococcus* spp. increasing in more severe lesions in mice infected with *L. major*. In humans, we found individuals with a dominance of *Staphylococcus aureus*, *Streptococcus* spp., or a mixture of both, although whether these distinct skin microbiota influences the outcome of disease is yet unknown. However, our studies in mice clearly suggest that further studies in patients are warranted.

Why dysbiosis occurs during cutaneous leishmaniasis, or in other inflammatory conditions, is unknown. Innate defenses, such as antimicrobial peptides (AMPs), can target certain bacteria and play a role in disrupting the microbiota in the intestine and in the skin (Cogen et al. 2010; Dorschner et al. 2001; Natsuga, Cipolat, Watt 2016; Nizet et al. 2001; Salzman et al. 2010), and may also be responsible for the dysbiosis caused by *L. major* infection. We found that infection with *L. major* causes changes in AMP expression in the skin (data

not shown), and mice deficient in a cathelicidin-type antimicrobial peptide (CAMP) appear more susceptible to infection with *L. amazonensis* (Kulkarni et al. 2006). Whether this deficiency in CAMP causes changes in the skin microbiota remains to be determined, but these results in addition to our own findings suggest that AMPs in cutaneous leishmaniasis warrant further investigation. How AMPs might promote these changes is unclear, but virulence factors can make bacteria resistant to AMPs and both *Staphylococcus* spp. and *Streptococcus* spp. express genes that protect them from AMP killing (Kristian et al. 2005; Peschel et al. 1999; Peschel et al. 2001), potentially providing them with a survival advantage during *L. major* infection.

One difficulty in studying the microbiota is assessing how changes in the skin microbiota influence disease, since skin dysbiosis is the consequence of the inflammatory response in the skin. While transmissibility of dysbiotic microbiota has been demonstrated in the intestinal tract (Elinav et al. 2011a; Zenewicz et al. 2013), our data is the first to demonstrate transmissibility of the skin microbiota in a murine model. In this study and previous studies, colonization with a single bacterial species enhanced pathology (Naik et al. 2012), and although this approach will be essential for dissecting how particular bacteria alter immune responses, it will not replicate the complex changes that might be associated with a naturally occurring dysbiosis. Our ability to generate a mouse with dysbiotic skin microbiota overcomes this issue, and has allowed us to demonstrate that a naturally acquired dysbiosis promotes increased inflammatory responses, and in the case of cutaneous leishmaniasis increased disease. It is not clear how this

transmission occurs, although consistent with our results, evidence from human studies indicates that the environment influences the skin microbiota (Song et al. 2013), and *L. major* infections in mice may provide a model to study the mechanisms involved.

The findings from our mouse model of cutaneous leishmaniasis are similar to the dysbiosis that occurs during human cutaneous leishmaniasis. Interestingly, the different topological sites of our samples did not show any differences in the skin microbiota, although we only had a few samples from moist and sebaceous sites. Yet comparable to what has been reported by culture dependent and independent methods (Isaac-Marquez and Lezama-Davila 2003; Sadeghian et al. 2011; Layegh et al. 2015; Salgado et al. 2016), our results demonstrated that *Staphylococcus aureus* and *Streptococcus* spp. are highly abundant on lesional skin. This dysbiosis was also present on skin sites adjacent to the lesion. However unlike our mouse model, the dysbiotic skin microbiota did not appear to be transmissible to contralateral skin sites. It is not yet clear why the dysbiosis is confined to the lesional and adjacent skin sites in human cutaneous leishmaniasis but it is likely to be due to differences in grooming and environmental conditions between mice and humans. However, the similarities in the dysbiotic microbiota between the mouse model and human cutaneous leishmaniasis demonstrate the utility of our model system to study the role of skin microbiota during leishmania infections.

One of our findings was that skin dysbiosis does not cause immunologic changes in the skin or disease by itself, nor did topical colonization with *S. xylosum*, similar to results reported by recent studies (Naik et al. 2015). However, in mice with a defective skin barrier induced by contact hypersensitivity to DNFB, *S. xylosum* exacerbated the inflammatory response, assessed by increased recruitment of neutrophils and upregulated expression of IL-1 $\beta$ . These results are consistent with other studies showing that mice with barrier defects allow *Staphylococcus* to penetrate the epidermal barrier and subsequently increase cytokine expression in the skin (Nakatsuji et al. 2016). In some situations the cytokine production may be protective, such as during a fungal infection (Naik et al. 2015). However, in cutaneous leishmaniasis, neutrophils and IL-1 $\beta$  are associated with increased pathology rather than the restriction of parasites (Charmoy et al. 2016; Fernandez-Figueroa et al. 2012; Gimblet et al. 2015; Gonzalez-Lombana et al. 2013; Novais et al. 2014a; Voronov et al. 2010). Thus, we hypothesize that *L. major* infection disturbs skin barrier integrity while simultaneously inducing a dysbiosis in the skin microbiota, which taken together leads to the increased recruitment of neutrophils and IL-1 $\beta$  recruiting cells to the skin, and causes increased lesion severity.

These results raise the obvious question of what role systemic or topical antibiotics might play in moderating inflammatory responses associated with leishmaniasis (Grice 2014). As previous studies with germ-free mice indicate that commensal bacteria may contribute to lesion severity in cutaneous leishmaniasis (de Oliveira et al. 1999; Naik et al. 2012; Oliveira et al. 2005), and our studies

demonstrate that dysbiosis exacerbates disease, it is reasonable to predict that antibiotic treatment might be beneficial in leishmaniasis. While we have been unsuccessful in moderating disease in mice by antibiotic treatment, there are examples of antibiotic therapy being protective in some cutaneous leishmaniasis patients (Aguilar et al. 2010; Ben Salah et al. 2013; Kim et al. 2009; Krolewiecki et al. 2002). However, there are other studies that find no effect of antibiotic treatment (Iraji and Sadeghinia 2005; Neva et al. 1997), and moreover when such treatment shows a positive outcome the mechanism involved is not clear. Given the different outcomes of studies looking at antibiotic treatment, and taken together with our results, it appears that the role of antibiotics in treatment needs further investigation.

In summary, our findings indicate that the skin microbiota not only changes during leishmania infection, but when transmitted to naïve mice can enhance disease to leishmania. These findings have obvious consequences when considering how to limit disease severity in cutaneous leishmaniasis. Moreover, since we find that the dominant bacteria associated with a leishmania-induced dysbiosis differs depending upon the severity of disease in mice, further epidemiologic studies with patients to determine the consequences of differing types of dysbiosis are warranted. Finally, we found that dysbiotic skin microbiota can be transmitted to conventional naïve mice, which provides a model to define how and when dysbiosis might influence control of other infections, autoimmune diseases and wound healing.

## CHAPTER 4: DISCUSSION

In this thesis we investigated whether IL-22 and the skin microbiota could influence disease during cutaneous leishmaniasis. Our results have demonstrated a role for IL-22 in limiting tissue damage, yet also demonstrates that dysbiosis in the skin microbiota exacerbates inflammation during infection. In both observations, expression of IL-1 $\alpha$  and/or IL-1 $\beta$ , cytokines that drive pathology during infection, were influenced by IL-22 and dysbiosis. Yet, changes in IL-22 expression or the skin microbiota composition did not influence the parasite burden, suggesting their roles in modulating disease during cutaneous leishmaniasis are mediated by modulating the immune response to promote wound healing or to exacerbate tissue damage. This chapter will discuss the implications and challenges of regulating IL-22 and the skin microbiota as therapies for cutaneous leishmaniasis; will discuss the utility of the dysbiotic mouse model to study other inflammatory skin diseases; and will posit potential ways the microbiota is changed during infection and how those changes may drive the immune response to cause pathology in the skin.

### **4.1 Implications of a dose-dependent requirement for IL-22 and areas for future investigation**

Our data suggest a role for IL-22 in limiting pathology during cutaneous leishmaniasis through initiating keratinocyte migration and decreasing the release of pro-inflammatory cytokines that exacerbate inflammation and tissue

damage. Yet, it was previously shown that IL-22 has no effect during a low dose infection with *L. major* (Brosch et al. 2014). Similarly, this thesis demonstrated that the protection provided by IL-22 was only observed with higher doses of the parasite, suggesting that a certain threshold of infection-induced inflammation is required for IL-22 to limit pathology. In fact, we observed that greater doses of parasites induced higher expression of IL-22 in the skin (Figure 17a). In the mouse model, higher doses of infection lead to the release of more inflammatory cytokines and subsequently more tissue damage. It is not yet clear how parasite burden dictates when IL-22 is required to limit protection or whether this dose-dependence is also observed in human patients. Data from this thesis provides the basis for future studies to investigate how this dose dependent requirement for IL-22 is mediated.

Previous studies speculated that IL-22 could have protective effects during leishmaniasis due to correlative studies in human patients (Pitta et al. 2009; Ghosh et al. 2013). However, these studies did not demonstrate a mechanism of how that protection is mediated. In this thesis, we demonstrated that in the absence of IL-22, mice infected with *L. major* had increased lesion sizes and lesion pathology. While the exact mechanism still needs further investigation, we observed aberrant expression of genes that regulate keratinocyte migration, keratin 6 and keratin 16, in the absence of IL-22. As keratinocyte mobility is required for wound healing (Haase et al. 2003), we hypothesize that this defect in keratinocyte mobility could delay lesion resolution during cutaneous leishmaniasis (Figure 17b). Surprisingly, we did not observe a difference in

keratinocyte proliferation or survival during infection with *L. major* in the absence of IL-22. But these observations were made using an intermediate dose of infection. Because our data demonstrates a dose-dependent requirement for IL-22, future studies could examine keratinocyte proliferation and survival during infection with varying doses of *L. major*. Due to increased inflammation with a high dose of infection, it is likely that more keratinocyte death is observed and the pro-survival effects of IL-22 become more apparent. These studies would provide more insight into how IL-22, parasite burden, and keratinocyte function contribute to disease resolution during cutaneous leishmaniasis.

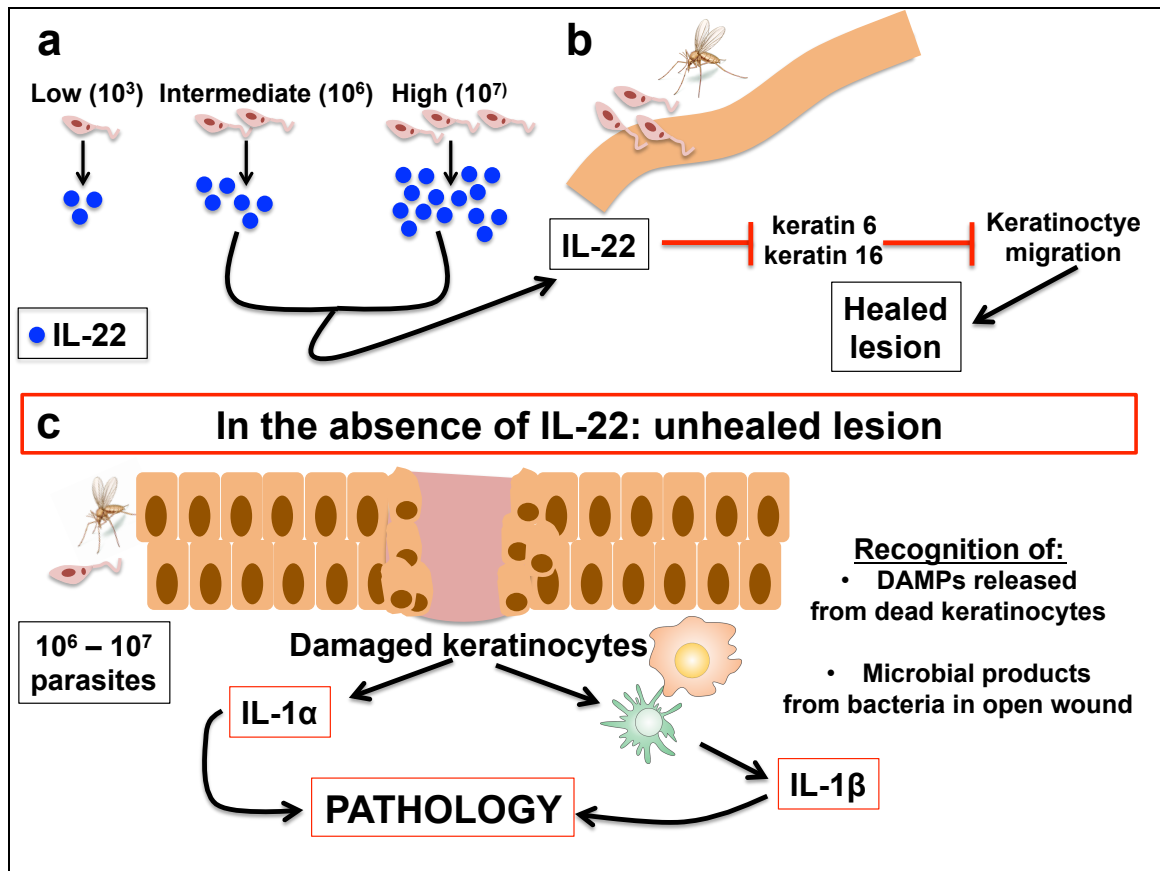
Keratinocytes release IL-1 $\alpha$  in response to injury or irritation in the skin (Kondo and Ohshima 1996; Spiekstra et al. 2005), which increases inflammatory cell recruitment into the skin. Simultaneously, the damaged keratinocytes could signal to innate cells to release damage-associated molecules like IL-1 $\beta$ . In the absence of IL-22, we observed increased expression of both IL-1 $\alpha$  and IL-1 $\beta$  during *L. major* infection (Figure 17c). IL-1 $\beta$ , in particular, is associated with neutrophil recruitment into the skin and increased pathology during cutaneous leishmaniasis (Fernandez-Figueroa et al. 2012; Novais et al. 2014a; Voronov et al. 2010; Gonzalez-Lombana et al. 2013; Charmoy et al. 2016). Excessive neutrophil recruitment and IL-1 $\beta$  release could also delay wound healing in the skin (Gutierrez-Fernandez et al. 2007; Goren et al. 2003; Mirza et al. 2013). Future studies could block IL-1 signaling and neutrophil recruitment in the absence of IL-22. These studies could determine if limiting this type of inflammation is another mechanism IL-22 uses to aid in tissue resolution,



Previous studies on IL-22 and leishmaniasis did not provide a clear understanding of the role of IL-22 during leishmania infections. Data from this thesis demonstrated that IL-22 limits tissue damage and aids lesion resolution during cutaneous leishmaniasis in a parasite dose-dependent manner. Future studies will provide more details on how parasite dose, inflammation, tissue damage, and IL-22 interact during infection with leishmania. With this information, we could better understand how to use IL-22 in the treatment of cutaneous leishmaniasis.

#### **4.2 Leishmania in the field of the skin microbiota**

In the field of the skin microbiota, some studies are only able to characterize how the microbiota changes throughout disease. In this thesis, we aimed to determine whether the microbiota changes during infection as well as understand what those changes could mean. We demonstrated that leishmania infection, in mice and humans, induces changes in the skin microbiota dependent upon the severity of the disease. This dysbiosis was characterized by high levels



**Figure 17: The role of IL-22 during *L. major* infection.** (a) IL-22 is induced during *L. major* infection in a dose dependent manner. (b) This production of IL-22 inhibits the expression of anti-migratory factors, keratin 6 and keratin 16, in keratinocytes. This blockade allows keratinocyte mobility and ultimately leads to wound closure and lesion resolution. (c) In the absence of IL-22, *L. major* lesions are larger and more severely ulcerated. This damage to the epithelial barrier could lead to the release of IL-1 $\alpha$  from damaged keratinocytes as well as signal to innate cells to produce IL-1 $\beta$  in response to DAMPs. Additionally, the open wound could become exposed to bacterial products, which would also prompt the release of IL-1 $\beta$ .

of *Staphylococcus* spp. or *Streptococcus* spp. in the community of bacteria present on the skin. These genera of bacteria, in particular *Staphylococcus* spp., have been associated with several skin disorders including atopic dermatitis, psoriasis, and chronic diabetic wounds (Gao et al. 2008; Alekseyenko et al. 2013; Kong et al. 2012; Price et al. 2009; Grice et al. 2010; Loesche et al. 2016;

Gardner et al. 2013). But the conclusions we can draw about these associations are limited due to the nature of some of the studies. Work presented in this thesis described that dysbiosis induced by leishmania infection does not induce inflammation on naïve skin, but after inflammation is initiated, either by *L. major* infection or contact hypersensitivity, the dysbiotic skin microbiota exacerbated inflammation and subsequent tissue damage. These data tell us that the skin microbiota is not only an effect of inflammation in the skin, but also plays an active role in disease.

Mono-colonization with a particular bacterium of interest has been used in several studies to determine if those bacteria play a role in disease. *S. epidermidis*, in particular, has been used to demonstrate that commensals can drive cytokine production from CD8+ T cells to protect against *C. albicans* infection (Naik et al. 2015). Additionally, mono-colonization with *S. epidermidis* primes regulatory T cells early after birth to limit inflammation during challenge with the bacterium later in life (Scharschmidt et al. 2015). These studies suggest that this common skin commensal is highly active in skin immunity. Similar observations were made during leishmania infection. Using a germ-free mouse model of cutaneous leishmaniasis, previous studies demonstrated that *S. epidermidis* drives inflammation and lesion development during *L. major* infection (Naik et al. 2012). These studies provided useful information about how a common skin commensal like *S. epidermidis* could influence the outcome of cutaneous leishmaniasis. However, there were limitations to these observations. We observed that during infection with leishmania parasites, *S. xylosum* and *S.*

*aureus* were dominant on the lesions of mice and humans, respectively. Our data suggests that *S. epidermidis* may be irrelevant during infection with leishmania. While mono-colonization can teach us about how the immune response interacts with common skin commensals, we believe it is important to study the commensals that are affected by inflammation in order to investigate how those changes influence disease. Utilizing the bacteria that naturally dominate during an inflammatory state might provide a better idea of interactions between the skin microbiota and the immune response during disease.

Recent studies demonstrated that in a mouse model of atopic dermatitis, *S. aureus* increased with disease, but also was important in driving inflammation (Kobayashi et al. 2015). Similarly, another study showed that a breakdown of skin integrity allowed *S. aureus* to translocate deeper into the skin and exacerbate inflammation (Nakatsuji et al. 2016). We believe these types of studies, as well as work presented in this thesis, go beyond characterizing the changes in the skin microbiota and begin to ask how those changes influence disease, potentially moving the field forward.

#### **4.3 Utility of the dysbiotic mouse model to study other inflammatory skin disorders**

Many studies have demonstrated associations with disease in the skin and a dysbiosis in the skin microbiota. Currently, our investigations into the skin microbiota are focused on determining if dysbiosis causes inflammation in other

inflammatory skin diseases. Some investigators, as well as our lab, have used mono-colonization of a particular bacterium to examine the effect of dysbiosis on disease and inflammation. However, there are limitations to this method. It typically requires a large amount of bacteria and multiple applications for a transient colonization. We established a model in these studies that could address some of these limitations. Uninfected mice co-housed with *L. major* infected mice acquired a similar dysbiosis characterized by a dominance of *S. xylosus*. While transferring dysbiosis by co-housing in other studies also transfers disease (Elinav et al. 2011b; Zenewicz et al. 2013) our model transferred the dysbiosis without inflammation in the skin, separating dysbiosis from disease. This system allowed us to investigate the role of dysbiosis prior to infection or disease. We believe this system could be useful in the study of other inflammatory skin diseases.

Our co-housing model of dysbiosis could allow us to test whether dysbiosis prior to injury exacerbates pathology in mouse models of psoriasis, atopic dermatitis, chronic diabetic wounds, or any other model of inflammatory skin disease. In our system, we used *L. major* infected mice to create dysbiosis on the skin of co-housed naïve mice. However, other inflammatory skin diseases could potentially be used to establish this goal. It is not yet known whether the dysbiosis caused in other inflammatory skin models is transferrable by co-housing. But some models induce similar, if not greater, inflammation than leishmania infection and thus could potentially use a co-housing system to test the importance of dysbiosis during infection. It would be interesting to determine

if other dysbiosis-causing commensals influence disease as *S. xylosum* does during infection with *L. major*. These studies could provide insight into which members of the microbiota are important in various skin diseases.

#### **4.4 Understanding how changes in the skin microbiota influence the immune response**

Our data demonstrates that leishmania infection causes changes in the skin microbiota and that acquiring a dysbiotic skin microbiota prior to infection magnifies inflammation and subsequent pathology in the skin. While these studies indicate that the skin microbiota plays a role in disease, it is not yet clear how these interactions are mediated.

We hypothesize that inflammation induced by infection initiates the changes in the microbiota that go on to increase immunopathology. Infection with leishmania parasites drives immune cell infiltrate into the skin, cytokine and chemokine production that magnifies that infiltration, and the production of antimicrobial molecules (Figure 18a). Molecules like reactive oxygen species (ROS) and nitric oxide (NO) are important in parasite control, but can also have anti-bacterial properties. Innate cells produce these molecules during infection with leishmania parasites, but this production mostly happens in the dermis of the skin, potentially not close enough to affect the bacteria on the surface.

Keratinocytes, on the other hand, are in the epidermis with direct access to surface bacteria. ROS and NO in the skin can be upregulated in keratinocytes in

response to cytokines like IFN- $\gamma$  (Bito and Nishigori 2012; Sur et al. 2002). While one study suggests that keratinocytes can produce NO during leishmania infection (Blank et al. 1996), it is unclear if this production plays any role in the change in the skin microbiota. The same is true for antimicrobial peptides (AMPs). During *L. major* infection, the expression of some AMPs was increased, while others decreased in expression in the skin (data not shown). There is evidence that lesions are more severe in the absence of the antimicrobial peptide, cathelicidin (Kulkarni et al. 2011), but whether the skin microbiota plays a role in this phenotype is unknown. Analyzing how the skin microbiota changes during infection in cathelicidin deficient mice as well as in other AMP deficient mice could inform us about the importance of these molecules in cutaneous leishmaniasis and the skin microbiota.

Our studies utilized the 16S ribosomal RNA gene to characterize how the skin microbiota changed throughout infection with leishmania. While this method was useful to identify the community of bacteria on the skin throughout infection, having more detailed information about how those bacteria change would be useful to better understand how the microbiota interacts with the host immune response. Our data describes the changes in the skin microbiota at the genus and species level, but it is clear that there are strain level differences in the skin bacterial communities that could stimulate the immune response differently (Fitz-Gibbon et al. 2013; Oh et al. 2014; Oh et al. 2016). For example, while some strains of *S. aureus* stimulated IL-17 production from  $\gamma\delta$  T cells through IL-1 $\beta$  signaling, other strains were unable to produce this reaction (Maher et al. 2013).

As IL-17 and IL-1 $\beta$  are known to drive pathology during cutaneous leishmaniasis, understanding strain differences could be important. We observed that *S. xyloso* and *S. aureus* was dominant on the lesions of leishmania infected mice and humans, respectively. However, these bacteria were also present on naïve and uninfected skin. We do not know if there are strain differences of bacteria on the skin before and after infection, but strain differences could influence bacterial gene expression and the type of host immune response initiated. Studies investigating how strains of bacteria change throughout disease could help us better understand dysbiosis and how it influences inflammation.

The bacteria that are a part of our microbiota also produce proteins and metabolites that can stimulate different immune responses. Short chain fatty acids (SCFAs) like butyrate, acetate, and propionate, have been shown to induce regulatory T cells, activate the inflammasome and IL-18 production, and regulate macrophage function (Smith et al. 2013; Macia et al. 2015; Chang et al. 2014). Indole, a tryptophan catabolite from microbiota, can engage the aryl hydrocarbon receptor (AHR) to induce IL-22 expression (Zelante et al. 2013).

Most studies have focused on intestinal metabolites and their interactions with the immune response. The bacterial metabolites of the skin have been less studied. However, recent studies have used 3D mass spectrometry mapping along with 16S rRNA gene sequencing to characterize the metabolites of the skin microbiota (Bouslimani et al. 2015). These studies demonstrate that members of the skin microbiota produce metabolites differently. During dysbiosis in



cutaneous leishmaniasis, it is likely that different metabolites are produced when certain bacteria become dominant. These metabolites could also be different between strains of bacteria that are present before and after infection. Recent studies have determined that there are strain-level differences in the production of metabolites from *Propionibacterium acnes*, a common skin commensal implicated as a pathogen of acne vulgaris. These studies suggest that different strains of *P. acnes* from healthy and diseased skin produce different levels of the metabolite, porphyrin, which may play a role in the pathogenesis of the disease (Johnson et al. 2016). Changes in the skin microbiota caused by leishmania infection may introduce different metabolites from the dominant bacteria that drive inflammation and subsequent pathology during cutaneous leishmaniasis (Figure 18b). Studies that examine the skin microbiota and their metabolites on healthy skin and how they change during disease are informative. But understanding the different roles metabolites play in modulating disease would be more beneficial. Future studies should examine how metabolites change during disease but also examine the interactions between bacterial metabolites and the immune system. In cutaneous leishmaniasis, where the pathology is largely mediated by the immune response, understanding the role of metabolites could be useful when considering how to regulate immunopathology.

Ultimately, future research on immune mediated diseases like cutaneous leishmaniasis should involve studying the immune response in addition to applying information from the genomics, transcriptomics, and metabolomics study of the microbiota. This type of research could build upon the work done in

this thesis and would provide a more complete picture of how the microbiota interacts with the immune system in disease.

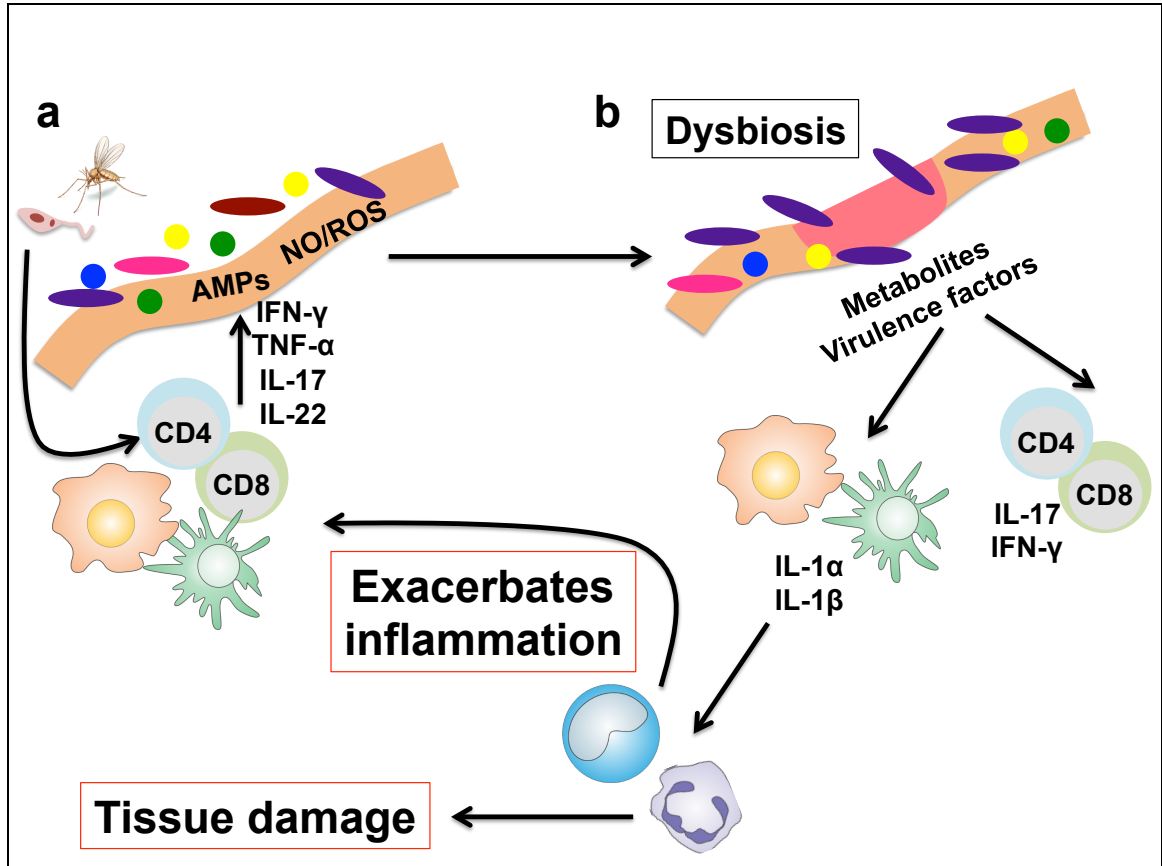
#### **4.5 Antibiotics and cutaneous leishmaniasis**

As our results have demonstrated that the skin commensal, *S. xylosus*, can exacerbate pathology in the skin during cutaneous leishmaniasis, using antibiotics in combination with anti-leishmania drugs might be a useful therapy.

There are several studies examining the effects of different antibiotics on cutaneous leishmaniasis, but the most studied has been Paromomycin.

Paromomycin is a protein synthase inhibitor that has been shown to increase lesion resolution during cutaneous leishmaniasis (el-Safi et al. 1990; Asilian et al. 1995; Ben Salah et al. 1995; Iraj and Sadeghinia 2005; Asilian and Davami 2006; Aguiar et al. 2010; Ben Salah et al. 2013). However just as many studies have demonstrated that paromomycin treatment has no effect or is less effective than current pentavalent antimony treatments (Hepburn, Tidman, Hunter 1994; Neva et al. 1997; Soto et al. 1998; Faghihi and Tavakoli-kia 2003; Armijos et al. 2004; Shazad, Abbaszadeh, Khamesipour 2005). These studies are complicated to interpret due to small study sizes, different species of the parasite as well as different methods of pentavalent antimony and antibiotic application (Kim et al. 2009). A well-controlled analysis is required to understand the most effective method to administer Paromomycin and if this treatment should be given in combination with drugs that also target the parasite.

Other antibiotics, like azithromycin, have also been reported to decrease lesion size and parasite burdens during cutaneous leishmaniasis (Krolewiecki et al. 2002), but more studies are needed to better understand how this protection



**Figure 18: The skin microbiota, dysbiosis, and leishmania infection.** (a) Infection with leishmania parasites causes an infiltration of immune cells into the skin that produce inflammatory cytokines. These cytokines can stimulate keratinocytes to produce ROS, NO, and AMPs in the epidermis. (b) The production of these antimicrobial factors initiates changes in the skin microbiota, which leads to a dominance of a particular bacterium. These bacteria can release metabolites and/or virulence factors, which modulate the immune response to exacerbate pathology.

is achieved. Even antifungals have been used to help lesions heal faster and decrease parasite burdens during cutaneous leishmaniasis (Pinheiro et al. 2016).

Amphoterin B is classified as an anti-fungal and anti-protozoan drug and has been effective as a topical treatment during cutaneous leishmaniasis (Ruiz et al. 2014). These antibiotics are believed to help during the resolution of leishmania infections by reducing parasite burdens. The mechanism of parasite control is not understood with the use of any of these antibiotics. Azithromycin can modulate macrophage function and can induce cytokine production from these cells (Krolewiecki et al. 2002; Xu et al. 1996; Ianaro et al. 2000). Thus, it can mediate anti-parasitic immunity by direct interactions with the immune response. Another possibility is that antibiotics modulate the skin microbiota, which indirectly shapes the type of immune response initiated during infection. Interestingly, there is evidence that Paromomycin can decrease bacterial contamination of leishmania lesions as well as decrease parasite burdens (el-On, Sneier, Elias 1992). It is possible that other antibiotics could also influence the bacteria on lesions during cutaneous leishmaniasis, but further characterization of the microbiota of leishmanial lesions after antibiotic treatment is needed to determine the effects of these drugs.

In agreement with work presented in this thesis, other studies have demonstrated that *Staphylococcus* spp. and *Streptococcus* spp. are significantly increased during cutaneous leishmaniasis (Layegh et al. 2015; Salgado et al. 2016). We hypothesize that utilizing antibiotics with some specificity for these two

genera might help alleviate disease. Current antibiotics tend to have a broad spectrum of targets and may not be ideal to target specific bacteria. Information from the species and strain levels of these bacteria may be required to generate antibiotics effective to treat cutaneous leishmaniasis. If such antibiotics are produced, a combined therapy of anti-leishmania and anti-staphylococcal or anti-streptococcal could be the most effective way to clear the parasite and regulate immune mediated pathology.

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