ABSTRACT

Listeria monocytogenes (LM) is a gram-positive bacterium and is a common contaminant in processed meats and dairy products. In humans, ingestion of LM can result in intracellular infection in the spleen and liver, which ultimately leads to septicemia, meningitis, and spontaneous abortion. Interleukin (IL)-23 is a cytokine that regulates immune responses by inducing the production of IL-17A, IL-17F, and IL-22, and is required for clearance of LM. IL-17A and IL-17F have been shown to recruit neutrophils to sites of infection, while IL-22 has been shown to induce secretion of anti-microbial peptides and has the ability to protect tissues from damage by preventing apoptosis. The role that IL-22 might play in LM bacterial clearance and resistance during an innate or adaptive immune response has not been thoroughly investigated. During infection, we have found that LM induced the production of IL-22, and during primary infection, IL-23 is required for IL-22 production. However, our findings suggest that IL-22 is not required for clearance of LM and does not seem to be required for the protection of the spleen and liver during a LM infection during a primary or secondary systemic or mucosal infection. Understanding the role of IL-22 will enable us to better understand the immune response against LM as well as similar pathogens. This knowledge will aid in the generation of effective vaccines against intracellular pathogens.

THE ROLE OF INTERLEUKIN-22 DURING SYSTEMIC AND MUCOSAL INFECTION WITH LISTERIA MONOCYTOGENES

THESIS

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CHAPTER I

INTRODUCTION

Listeria monocytogenes

Listeria monocytogenes (LM) is an intracellular, gram-positive bacterium found in soil and water and is a common contaminant in processed meats and dairy products. Once ingested by humans, Internalin A on the surface of LM binds with human epithelial cadherin (E-cadherin) in the intestine, allowing the bacteria to pass through the intestinal epithelium and into the bloodstream. LM then travels to the spleen and liver, resulting in intracellular infection of several different cell types. LM infection can ultimately cause septicemia and meningitis in immunocompromised individuals. Women who are pregnant can develop chorioamnionitis if infected with LM, which can cause spontaneous abortion [1]. Due to a single amino acid change in E-cadherin in mice, LM is not able to efficiently adhere to the epithelial layer and is thus not able to easily pass through the intestine [1]. This can be overcome by infecting mice through the mucosal intra-gastric (i.g.) route with high doses of LM [2-4] or using another route of infection. Systemic intravenous (i.v.) infection of mice directly into the blood allows LM to disseminate into the spleen and liver, bypassing the intestines [4]. Invasion into the host cell cytoplasm requires virulence factor listeriolysin O (LLO), which destroys the host cell vacuole and allows LM to

escape. Once in the cytoplasm, actin-assembly- inducing protein (ActA) allows the LM to utilize host actin and propel itself to adjacent cells for infection [1].

Innate immune response to LM

Innate immune responses are composed of a variety of different cell types and are the immediate defenses against pathogens invading the host organism. During LM infection, the bacteria interact with pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). TLR 2 and 5 recognize peptidoglycan, lipoproteins, and bacteria flagella and signal through the adaptor protein molecule myeloid differentiation primary-response protein (MyD88) to activate the innate immune system [1;5]. During an oral model of infection, LM can also induce signaling through nucleotide-binding oligomerization domain (NOD) proteins that are located in the intestine to activate the immune system [6]. Mice require T cells to ultimately clear LM infection, however, innate immune mechanisms initially control the bacterial growth and resistance to infection [1;7;8]. Interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) are required for innate clearance of LM infection, and mice lacking these cytokines or their receptors are highly susceptible to LM and die at early time points [9-13]. To clear LM, IFN-γ is produced by natural killer (NK) cells and CD8+ T cells and is able to activate macrophages which are able to directly kill LM. Neutrophils are also able to kill LM using oxidative burst and nitric oxide [1;14-16]. The innate immune system is able to contain a LM infection in tissues, while being able to tailor the adaptive immune response.

Adaptive immune response to LM

Adaptive immune responses are specific immune responses against a pathogen. Immune cells, such as T and B cells, are activated during a primary infection, and are important for memory during re-infection. When the host is re-infected with the pathogen, T and B cells are able to quickly respond to the invading pathogen. For clearance of LM during an infection, antigen presenting cells, such as dendritic cells, macrophages and neutrophils, prime CD8+ T cells [17-19]. Dendritic cells are the optimal antigen presenting cell for inducing a CD8+ T cell response, giving a more robust expansion of CD8+ T cells. CD8+ T cells are important due to their ability to kill infected cells and secrete cytokines and chemokines such as IFN-γ, TNF-α, and CCL3 to activate and recruit more inflammatory cells to sites of infection[20;21]. B cells only play a small role against LM infection, though antibodies against LLO have been implicated in decreased susceptibility to LM infections [22].

Interleukin-23

Interleukin (IL)-23, a member of the IL-12 family of cytokines, shares a p40 subunit with IL-12 but is also comprised of a unique p19 subunit [23;24]. IL-23 binds to a receptor complex on lymphocytes that contain the IL-12 receptor-β1 and a novel IL-23 receptor [23]. IL-23 is secreted by macrophages and dendritic cells in response to invading pathogens [25] and is known to have both a protective and pathogenic function. IL-23 has been implicated to have a pathogenic role in autoimmunity and allergies [26-28]. IL-23 plays a protective role mainly against extracellular or vacuole-bound pathogens such as *Klebsiella pneumoniae* [29;30], *Citrobacter rodentium* [31;32], and *Salmonella enterica* [33;34], as well as against infections with *Toxoplasma gondii* [35;36] and *Candida albicans* [37-39]. Although IL-23 is in the IL-12 family, IL-23 does not have the same functions as

IL-12. IL-23 expands and maintains IL-17 secreting T cells, which are known to secrete IL-17A, IL-17F, and IL-22 [40]. Our lab has shown that during LM infection, IL-23 is required for the production of IL-17A and IL-17F [41]. This IL-23/IL-17 axis is required for clearance of LM from the spleen and liver. Our lab has previously published that when either IL-23 or the IL-17 receptor A, the shared receptor for IL-17A and IL-17F, were absent during i.v. LM infection, there was lower recruitment of neutrophils to the liver, but not the spleen. This suggests that IL-23 and IL-17 receptor A are required for the optimal recruitment of neutrophils to the liver, but not the spleen, during a primary i.v. infection with LM [41;42]. The mechanism by which IL-23 offers protection against LM in the spleen, however, remains unknown. IL-23 induced production of IL-22 is one possible mechanism.

Interleukin-22

IL-23 can also directly induce the production of IL-22 [32] which has been found to be produced by T cells, natural killer (NK) cells, NK T cells, and lymphoid tissue inducer (LTi) cells [43]. In certain infectious models, the production of IL-22 is regulated by IL-23 and has been shown to have the ability to induce the production of antimicrobial peptides [40]. During *Citrobacter rodentium* infection, IL-22 induces the production of Reg family and S100 family of antimicrobial peptides [32]. During *Klebsiella pneumoniae* infection, IL-22 produced by CD4+ T cells regulates lipocalin which is able to inhibit bacterial growth by sequestering iron [44]. IL-22 is also capable of inducing the production of lipopolysaccharide-binding protein and acute phase serum amyloid A [43;45;46]. While the role of IL-22 and its production of antimicrobial peptides during LM infection remains unstudied, LM is known to be killed by antimicrobial peptides including RegIIIγ, protegrins, ubiquicidin, cryptidins (α-defensins), and cathelin-related

antimicrobial peptides (CRAMP) [47-51]. At day 3 post LM infection, it has been found that IL-22 is not required for clearance of LM from the spleen and liver during a systemic i.v. infection [52]. However, the role of IL-22 at other time points during a systemic i.v. or mucosal i.g. LM infection has not been studied. Other models have also shown a role for IL-22 in protecting tissues against damage. In the *Klebsiella pneumoniae* model, production of IL-22 was able to protect lung tissue [44]. IL-22 can also protect hepatocytes against acute liver inflammation [52]. During a primary LM infection, mice lacking IL-22 may have more tissue damage. If these mice lacking IL-22 have tissue damage during a primary LM infection, there maybe higher bacterial burdens compared to wild type mice. While the ability of IL-22 to induce antimicrobial peptides and protect against tissue damage has been shown to be protective in infectious models, IL-22 has also been implicated in autoimmune diseases. Patients with Crohn's disease, an inflammatory bowel disease, had high levels of IL-22 in the blood and in lesions on the intestine [53-55]. IL-22 has also been shown to play a role in psoriasis, an autoimmune disease that causes skin lesions [56-59].

IL-23, IL-17A, and IL-17F have been implicated in recruiting neutrophils to the liver during LM infection, leading to enhanced clearance of LM [41]. However, the mechanism of IL-23 for LM clearance in the spleen has not been identified. IL-22 is a downstream cytokine from IL-23 and is known to induce the secretion of antimicrobial peptides as well as protect tissues from damage, and therefore could be a mechanism to clear LM from the spleen and liver. During LM infection, the factors involved in the regulation of IL-22 secretion as well as the role that IL-22 might play in bacterial clearance and resistance during an innate or adaptive immune response have not been thoroughly investigated. We hypothesize that IL-22 plays a role during primary and secondary LM infection by clearing LM from infected tissues, or by protecting tissues from

damage. We will test this hypothesis with the following specific aims: 1) To investigate the role of IL-22 during primary and secondary **systemic i.v. LM infection** by determining bacterial clearance and protection of tissues, and 2) to determine the necessity of IL-22 in the clearance of LM from the spleen, liver, and intestine during a primary and secondary **mucosal i.g. LM infection.**

Significance

These experiments look at the effect of IL-23 and its downstream cytokine, IL-22, as part of the immune response against intracellular pathogenic microorganisms. By studying disease mechanisms, especially against pathogens, we can provide future strategies that can be used to control diseases. Also, IL-22 has been shown to be elevated in autoimmune diseases, so understanding the effects of this cytokine can lead to possible treatments. An oral model of infection that mimics the natural route of infection in humans will lead to a better understanding of how to control LM outbreaks in the population.

CHAPTER II

METHODS AND MATERIALS

Mice

C57Bl/6 (B6) mice were purchased from Charles River. IL-23p19 knock out (IL-23 KO) and IL-22 KO mice on a B6 background have been previously described [60;61]. Male and female mice between 5 to 12 weeks of age were used. Mice were housed with food and water ad libitum in sterile microisolator cages with sterile bedding at the University of North Texas Health Science Center American Association for the Accreditation of Laboratory Animal Care accredited animal facility. All animal studies were performed under the approval of the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

Listeria monocytogenes infections and quantification of bacterial burden

Listeria monocytogenes (LM) 10403s was grown on brain-heart infusion (BHI) agar plates (BD Bacto) and virulent stocks were maintained by passage though B6 mice every two months. Streptomycin resistant LM (LM/strep^r) was generated as described in Chapter IV. For infection of mice, log-phase cultures of LM or LM/strep^r were grown in BHI broth, washed twice, and diluted in PBS to the desired concentration. For systemic intravenous (i.v.) infections, unless otherwise stated, mice were injected with ~1x10^4 LM via the lateral tail vein for a primary

infection. For a secondary systemic i.v. infection, mice were i.v. infected with ~$1x10^3$ LM, then allowed six weeks to recover and clear primary infection before being i.v. re-infected with ~$1x10^6$ LM. For mucosal intragastric (i.g.) infection, unless otherwise stated, mice were injected with ~$1x10^7$ LM/strep^r via the esophageal cavity using a gavage needle for a primary infection. For a secondary mucosal i.g. infection, mice were i.g. infected with ~$1x10^7$ LM/strep^r, then i.g. re-infected six weeks later with ~$1x10^8$ LM/strep^r. Mice were fasted at least 4 hours prior to i.g. infection to prevent food in the digestive tract from causing LM to be aspirated into the lungs [62]. To determine LM colony forming units (CFUs), spleens and livers from infected mice were homogenized in sterile double distilled H_2O. Small intestines from i.g. infected mice were extracted by cutting below the stomach and above the cecum, flushed with PBS to remove debris, and homogenized in sterile double distilled H_2O. Whole blood was collected into tubes containing HBSS with heparin and was centrifuged at 14000 rpm for 3 min. After removing the supernatant, the pellet was resuspended in sterile double distilled H_2O. Serial dilutions (1:10) of the tissues were prepared and 50 µl of each dilution was plated on BHI agar plates. After overnight incubation at 37°C, colonies were counted and LM CFUs recovered from each tissue were calculated.

In vitro procedures

Serum was obtained by removing the supernatant from whole blood following centrifugation at 14000 rpm for 30 min. For experiments using splenocytes for culture or ex vivo stain, spleens were homogenized with frosted microscope slides and red blood cells were lysed in Tris-ammonium chloride. Splenocytes were cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Atlanta Biologicals), L-glutamine, vitamins,

penicillin/streptomycin, nonessential amino acids, and sodium pyruvate. All supplements were from Invitrogen-Gibco. Splenocytes were cultured in the presence of heat-killed LM (HKLM) with a multiplicity of infection of 50:1. Cell culture was performed at 37°C in humidified air containing 5% CO_2.

IL-22 ELISA

ELISAs were performed on serum or filtered, cell-free splenocyte supernatant. Quantification of IL-22 was performed using antibodies from PeproTech. Cytokine levels were determined by comparison with standard curves generated from recombinant IL-22 (PeproTech) and were analyzed using a Biotek EL808 spectrophotometer.

Annexin V staining and alanine aminotransferase

Staining of splenocytes involved incubation at 4°C for 15 min with saturating amounts of CD45.2 FITC (BD Pharmingen) and Fc block (Pharmingen). Cells were then resuspended in binding buffer (BD Pharmingen) and Annexin V PE (BD Pharmingen) was added. Data was acquired and analyzed within an hour of staining using a Beckman Coulter Cytomics FC500. Serum alanine amino transferase (ALT) levels were quantified by colorimetric ALT enzyme assays (Biotron Diagnostics Inc.) according to manufactures directions.

Immunohistochemistry and microscopy

Immunohistochemistry of spleens and livers was performed by making 5-µm sections of frozen spleens and livers from LM infected B6 and IL-22 KO mice using a Leica CM 1850 cryostat. Spleen and liver sections were acetone fixed for 10 minutes before staining. Slides were washed

three times by submersion in PBS. To block tissue sections, slides were incubated for 45 minutes with 300 μL of 1X PBS+0.1% BSA + 0.1% sodium azide. After washing, the slides were incubated with purified anti-Ly6G (1A8) (BD Pharmingen) and Difco Listeria O polyserum (Fisher Scientific) for 45 minutes in filtered 1X PBS+0.05% Tween-20. The anti-Ly6G antibody was developed with anti-rat Alexafluor 594 (Molecular Probes) and the Difco Listeria O polyserum was developed with anti-rabbit Alexafluor 488 (BD Pharmingen) for 45 minutes in filtered 1X PBS+0.05% Tween-20. Prolong Gold antifade reagent (Invitrogen) and a cover slip were added to the stained tissues. To view the stained tissue, an Olympus Ax70 fluorescence microscope was used and images were captured with an Olympus DP70 digital camera.

Statistical analysis

Analyses of variances (ANOVAs) or student t tests were conducted on the data where appropriate. Bonferroni t-tests and Tukey-Kramer analyses were used for post-hoc analyses. LM CFU data was log transformed prior to analysis, and is represented as such in the figures. A p value of 0.05 or less was considered significant in all cases.

CHAPTER III

THE ROLE OF IL-22 DURING SYSTEMIC LM INFECTION

LM induces IL-22 production and IL-22 production requires IL-23 during systemic i.v. infection

Previous research from our lab has shown that IL-23 is required for survival and clearance of LM from the spleen and liver during a primary systemic LM infection [41]. However, the mechanism by which IL-23 is able to influence clearance of LM from the spleen is unknown. IL-23 has been shown to be required for the optimal production of IL-17A, IL-17F, and IL-22 in other models of infection [41]. To determine if IL-22 is produced during LM infection, serum and spleens were harvested from uninfected B6 mice or B6 and IL-23 KO mice that were i.v. infected with $1x10^4$ LM for 3 days. The concentration of IL-22 was measured in the serum and splenocyte culture supernatants stimulated with or without heat-killed LM (HKLM) or IL-23 using ELISA. LM infection induced IL-22 production in B6 mice in both the serum (**Figure 1A**) and the spleen (**Figure 1B**) as compared to uninfected B6 mice. In IL-23 KO mice, the amount of IL-22 was reduced in the serum and splenocyte culture supernatant with or without HKLM compared to LM infected B6 mice (**Figure 1A, 1B,** and **1C**). Therefore, LM infection induces the production of IL-22 and the production of IL-22 is dependent on IL-23 during a primary systemic i.v. infection. However, there were no differences in IL-22 production in splenocytes

re-stimulated with IL-23 (**Figure 1D**). This suggests that the cells capable of producing IL-22 are still present in IL-23 KO mice, but are not able to secrete IL-22 without the presence of IL-23. A similar pattern of IL-22 secretion was observed at days 5 and 7 p.i., as well as in the liver (data not shown).

IL-22 is not required for survival or clearance of LM during systemic i.v. infection

IL-22 has been shown to induce the production of antimicrobial peptides which could lead to a decrease in LM bacterial burden, causing an increase in survival. To determine if IL-22 has a role during a systemic infection, B6 and IL-22 KO mice were i.v. infected with $3x10^4$ LM for a survival study. Mice were weighed and observed for 12 days. There were no differences in survival between B6 and IL-22 KO mice (**Figure 2**), as well as no differences in weight loss (data not shown) during LM infection, suggesting that IL-22 is not required for survival during a systemic i.v. LM infection.

Although no differences were observed in survival between B6 and IL-22 KO mice, we wanted to determine if IL-22 impacted the clearance of LM from the spleen or liver during a systemic i.v. infection. For a primary infection, B6 and IL-22 KO mice were infected with LM and at days 1, 3, 5, and 7 post infection (p.i.) spleens and livers were harvested and bacterial burdens were determined. There were no differences in LM CFUs between B6 and IL-22 KO at days 1, 3, 5, and 7 p.i. (**Figure 3**). These data suggest that IL-22 is not required for clearance of LM from the spleen or liver during a primary systemic LM infection. However, these data do not preclude IL-22 from having a role during a secondary systemic LM infection. To determine if IL-22 impacts the clearance of LM after a secondary exposure to the pathogen, B6 and IL-22 KO mice were re-infected six weeks after a primary LM infection. Spleens, livers, and blood were

harvested at day 2 post secondary infection and bacterial burdens were determined in these tissues. During a secondary infection, there were no differences in LM CFUs between B6 and IL-22 KO mice in the spleen, liver, or blood (**Figure 4**). These data suggest that IL-22 is not required for optimal bacterial clearance during a primary or secondary systemic i.v. infection. Since there was no difference in LM bacterial burden during a secondary infection, IL-22 does not seem to be required to prime T cells during a primary systemic i.v. infection, as previously suggested [52].

IL-22 production is dependent on IL-23 during a primary systemic LM infection (**Figure 1**). We were interested in determining if IL-22 production was dependent on IL-23 during a secondary systemic i.v. LM infection. B6 and IL-23 KO mice were infected, then re-infected six weeks later. Spleens were harvested at day 2 p.i. and splenocytes were cultured overnight. The production of IL-22 was measured with ELISA in splenocyte culture with or without HKLM or IL-23. Unlike what was observed in the primary systemic LM infection, IL-22 was produced in IL-23 KO mice and there was no difference between B6 and IL-23 KO (**Figure 5A, 5B, 5C,** and **5D**). This suggests that IL-23 is required for IL-22 production during a primary systemic i.v. LM infection (**Figure 1A, 1B,** and **1C**) but a factor other than IL-23 is able to induce the production of IL-22 during a secondary systemic i.v. infection.

IL-22 is not required for protection of tissue during systemic i.v. LM infection

While our data suggest that IL-22 is not required for bacterial clearance during systemic LM infection, other models have found that IL-22 protects against apoptosis[44;52]. IL-22 secreted from lymphocytes binds to the IL-22 receptor on non-hematopoietic tissue cells, protecting these cells from damage [44;63;64]. When mice were stimulated with ConA, more liver damage was seen

in IL-22 KO mice than in control mice [52]. The ability of IL-22 to prevent apoptosis during LM infection is unknown. To investigate the role of IL-22 in apoptosis during a primary systemic LM infection, spleens were harvested from LM infected B6 and IL-22 KO mice at days 1, 3, and 5 p.i. Determination of the requirement of IL-22 for tissue protection in the spleen was accomplished by staining splenocytes with Annexin V to be analyzed by flow cytometry. Annexin V binds to cell membranes that have undergone apoptosis. During a primary infection, there were no differences in the percentage of cells undergoing apoptosis in the spleens between B6 and IL-22 KO mice at days 1, 3, and 5 p.i. (**Figure 6A, 6B,** and **6C**). In order to determine if epithelial cells are undergoing apoptosis, splenocytes were also stained with CD45, which binds to hematopoietic cells. Therefore, spleen epithelial cells that have undergone apoptosis will be positive for Annexin V and negative for CD45. There were no differences in the percentage of epithelial cells undergoing apoptosis between B6 and IL-22 KO mice during a primary infection (**Figure 6D, 6E,** and **6F**). In order to investigate whether or not IL-22 protects splenic tissue from apoptosis during a secondary exposure to LM, B6 and IL-22 KO mice were re-infected with LM and spleens were harvested at day 2 post secondary infection. Splenocytes were stained with Annexin V and CD45 to determine apoptosis. During a secondary infection, there were no differences in the percentage of apoptotic cells in spleens between B6 and IL-22 KO (**Figure 7A**). Also, there were no differences in the percentage of splenic epithelial cells undergoing apoptosis between these two strains of mice during a secondary systemic i.v. LM infection (**Figure 7B**).

In order to more thoroughly investigate the role that IL-22 might be playing in preventing tissue damage during LM infection, liver damage was assessed by measuring alanine aminotransferase (ALT) in the serum. ALT is stored in the liver, but in the presence of liver

damage, ALT is released into the blood and can be detected in the serum [52;65]. For primary systemic i.v. infections, B6 and IL-22 KO mouse serum was harvested at days 1 and 3 p.i. For a secondary infection, B6 and IL-22 KO mouse serum was harvested at day 2 post secondary infection. There were no differences in serum ALT levels between B6 and IL-22 KO mice, and this was seen in both primary (**Figure 8**) and secondary (**Figure 9**) systemic i.v. LM infection. This suggests that IL-22 does not play a role in protecting tissues from damage during systemic i.v. LM infection.

In order to visualize LM induced damage of B6 and IL-22 KO mice during systemic LM infection, infected spleens and livers were stained for the presence of neutrophils and LM using immunohistochemistry. For a primary systemic infection, spleens and livers from B6 and IL-22 KO mice were harvested at day 3 p.i. In the spleens of both B6 and IL-22 KO mice, we observed dissemination of LM throughout the organ. Although we were not able to discern if the damage was due to LM infection, there do not appear to be any differences between B6 and IL-22 KO mice at day 3 post systemic LM infection (data not shown). In the livers, lesions of LM and neutrophils were observed in both B6 (**Figure 10A**) and IL-22 KO (**Figure 10B**) mice. There do not appear to be any differences between B6 and IL-22 KO mice at day 3 post LM infection. We also did not note any differences in the number and size of LM lesions between B6 and IL-22 KO mice. During a secondary infection, the livers displayed similar LM and neutrophil lesions as the primary mice, and there were no differences between B6 (**Figure 11A**) and IL-22 KO (**Figure 11B**) mice. Again, we did not note any differences in the number and size of LM lesions between B6 and IL-22 KO mice. The spleens of secondary systemic LM infected B6 and IL-22 KO mice both lacked LM within the tissue, but splenic follicles were intact (data not shown). This suggests that during a secondary systemic LM infection, the spleens are able to clear LM

quickly and sustain little tissue damage. Collectively, the data from apoptosis assays, ALT assays, and immunohistochemistry suggest that IL-22 is not required for optimal tissue protection during a primary or secondary systemic LM infection.

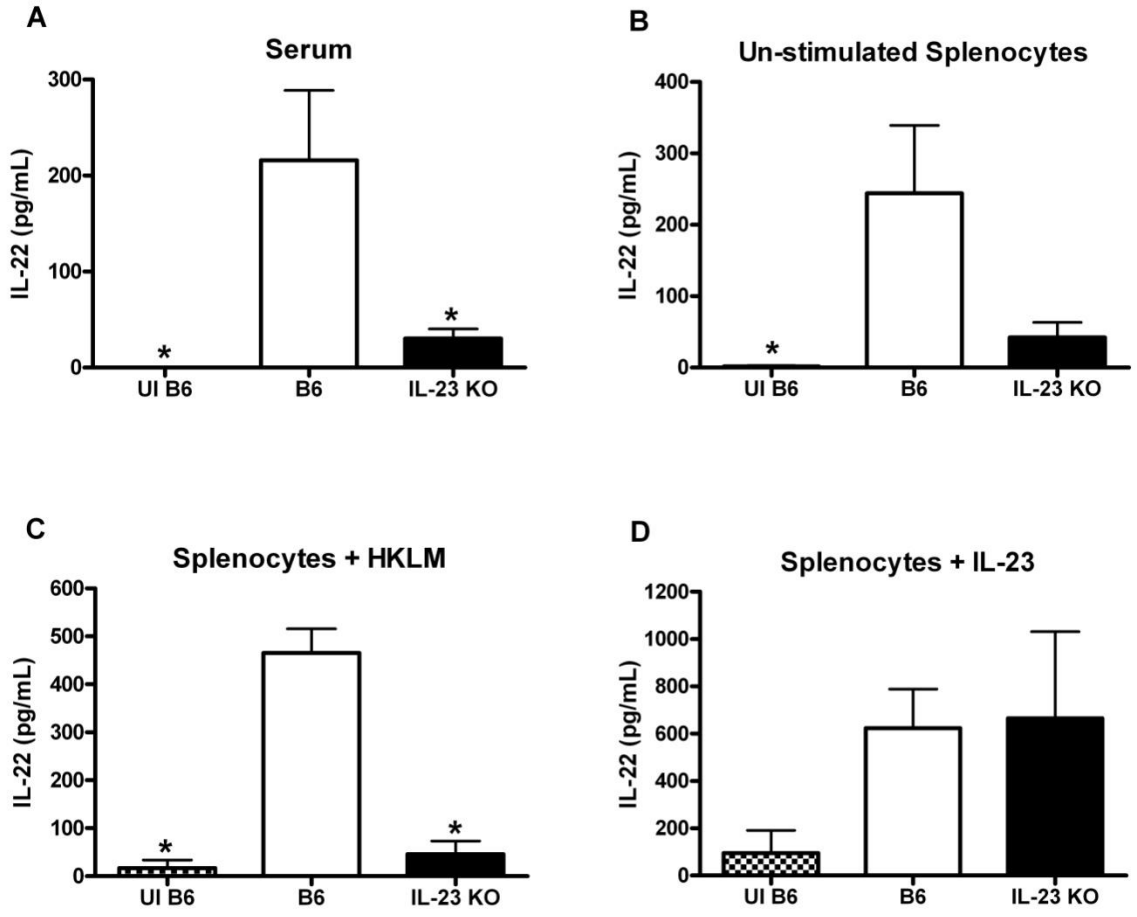

Figure 1. IL-22 is induced during systemic LM infection and requires IL-23.

Serum and spleens were harvested from uninfected B6 mice (UI B6) and B6 and IL-23 KO mice i.v. infected with $1x10^4$ LM for 3 days. IL-22 concentration was measured using ELISA in serum (A) and splenocyte culture supernatants from overnight un-stimulated (B), HKLM (C), or IL-23 (D) stimulated cultures. One-way ANOVAs detected significant effects of mouse strain ($\underline{p} \leq 0.05$). An * indicates a significant difference from B6 ($\underline{p} \leq 0.05$). All data are expressed as the mean $\pm$ SEM (n = 5/group).

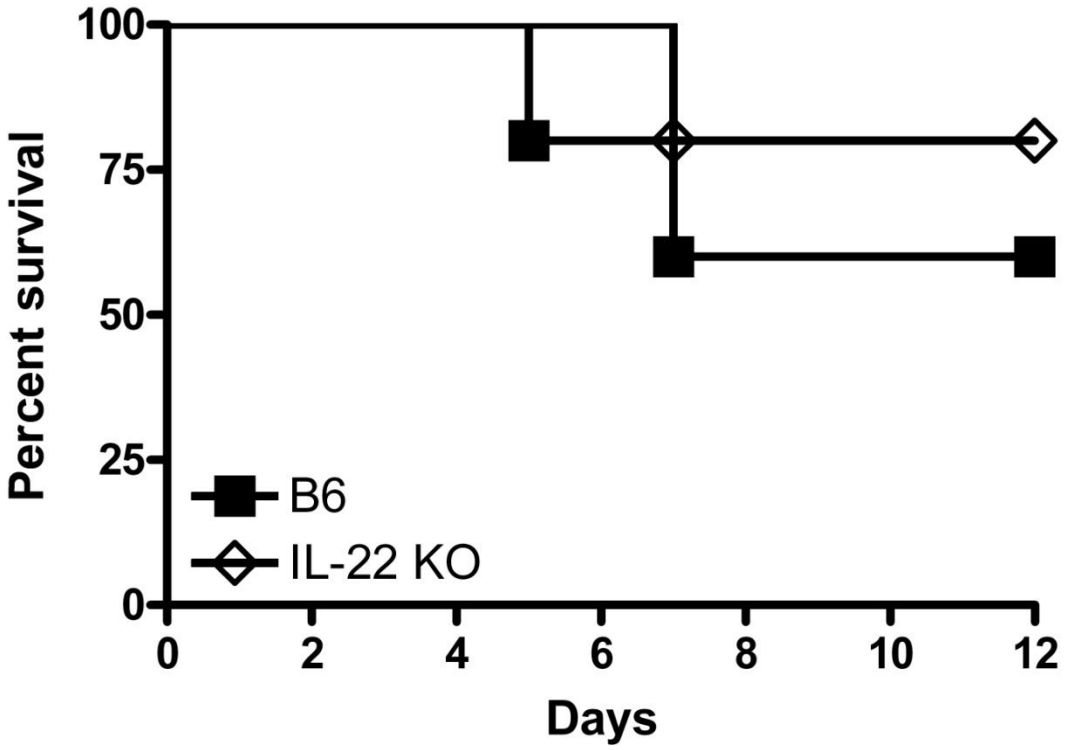

Figure 2. IL-22 is not required for survival during a systemic LM infection.

B6 and IL-22 KO mice were i.v. infected with $3x10^4$ LM for a survival study. A log rank analysis did not detect a significant difference between the survival curves of B6 and IL-22 KO mice, ($p \geq 0.05$), (n=5/group).

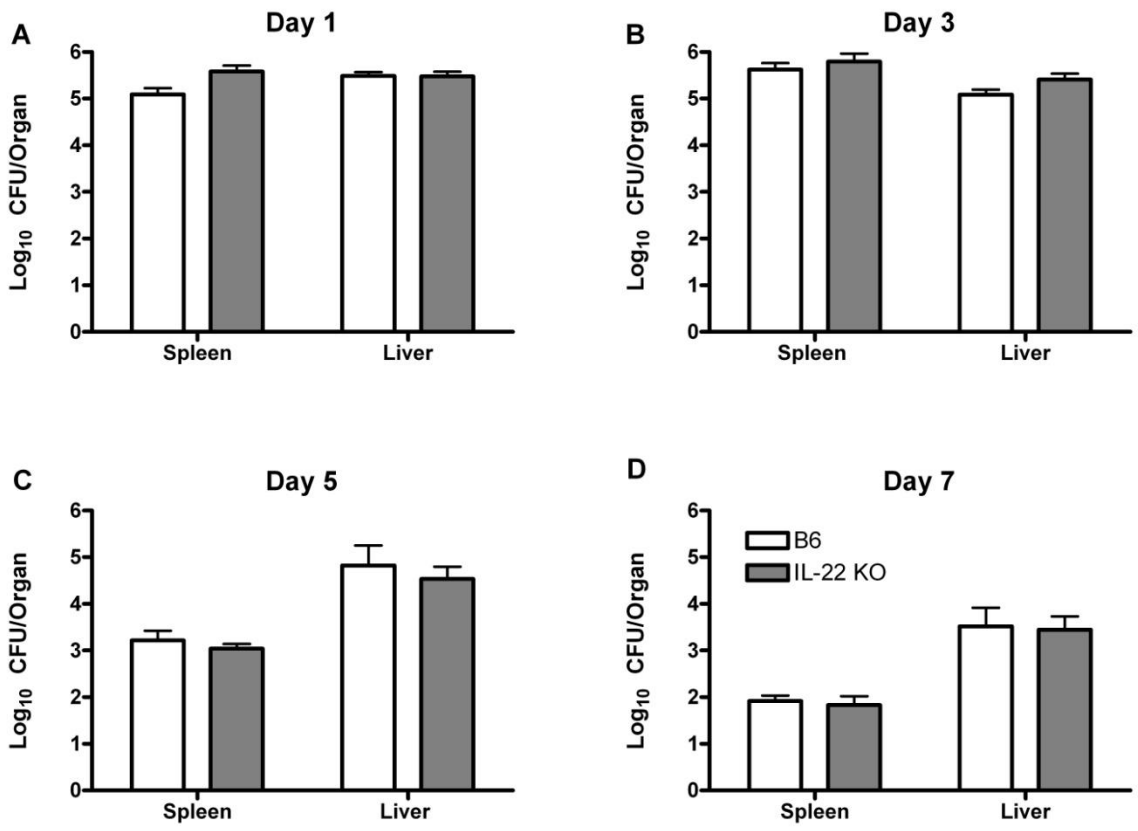

Figure 3. IL-22 is not required for clearance of bacteria from the spleen and liver during a primary systemic LM infection.

B6 and IL-22 KO mice were i.v. infected with $1x10^4$ LM. Spleens and livers were harvested 1, 3, 5, and 7 days p.i. and bacterial burdens were determined. Two-way ANOVAs did not detect significant effects of mouse strain ($p \geq 0.05$). These data are combined from two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 10/group).

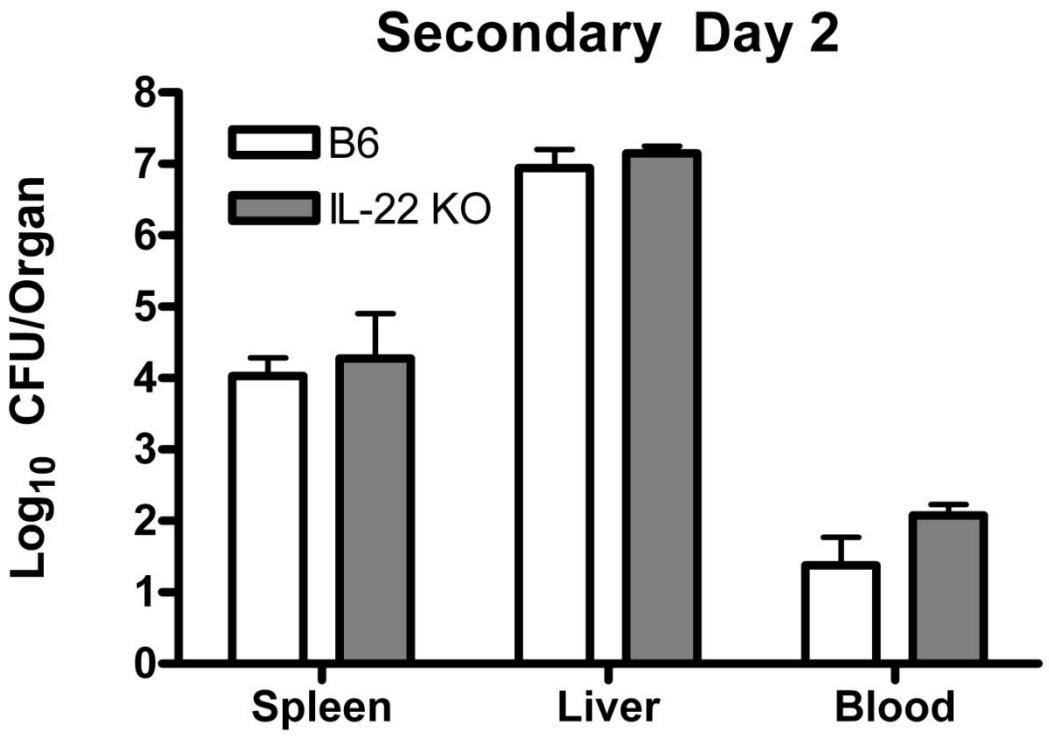

Figure 4. IL-22 is not required for clearance of bacteria from the spleen, liver, and blood during a secondary systemic LM infection.

B6 and IL-22 KO mice were i.v. infected with $1x10^3$ LM, then re-infected six-weeks later with $1x10^6$ LM. Spleens, livers, and blood were harvested 2 days p.i. and bacterial burdens were determined. A two-way ANOVA did not detect a significant effect of mouse strain ($\underline{p} \geq 0.05$). These data are combined from two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 9/group).

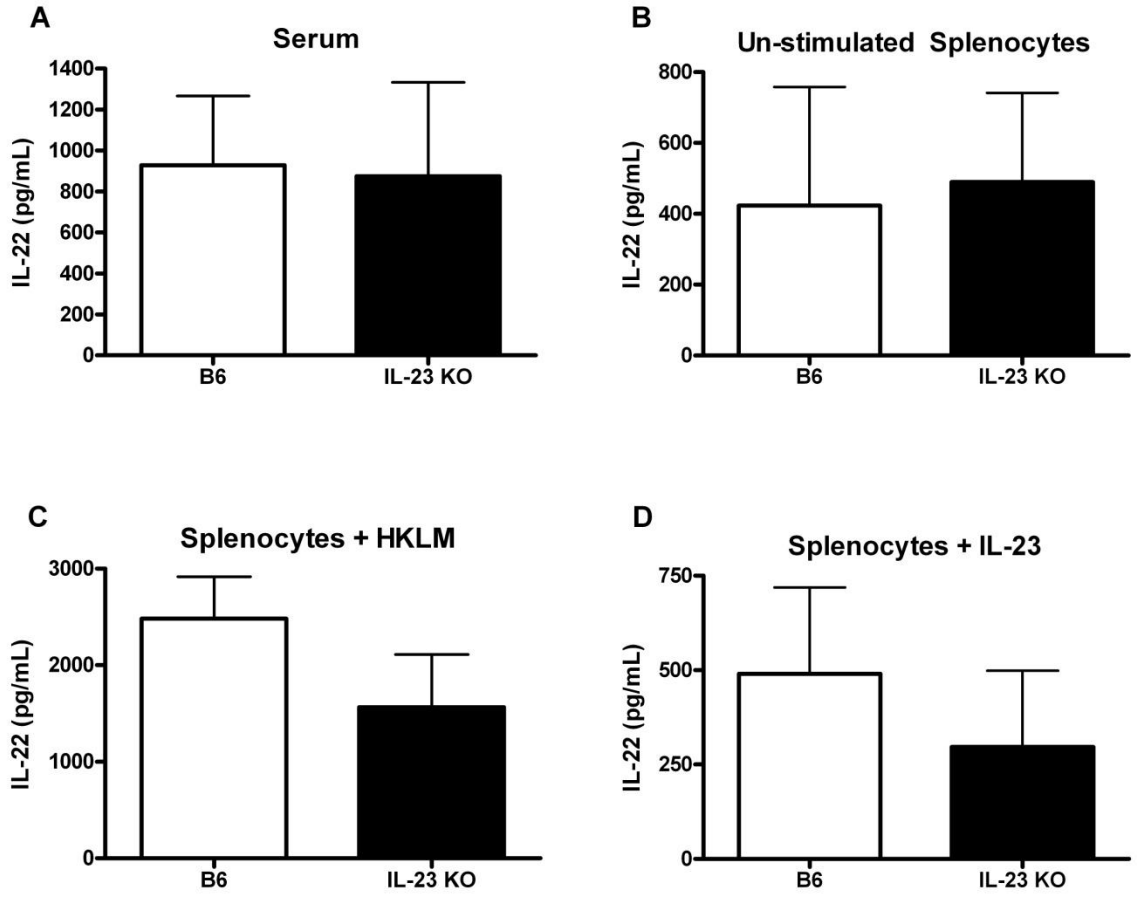

Figure 5. IL-23 is not required for IL-22 production during a secondary systemic LM infection.

B6 and IL-23 KO were i.v. infected with $1x10^3$ LM, then re-infected six-weeks later with $1x10^6$ LM. Serum and spleens were harvested 2 days p.i. IL-22 concentration was measured using ELISA in serum (A) and splenocyte culture supernatants from un-stimulated (B), HKLM (C), or IL-23 (D) stimulated cultures. t-test did not detect significant effects of mouse strain ($p \geq 0.05$). These data are representative of two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 4/group).

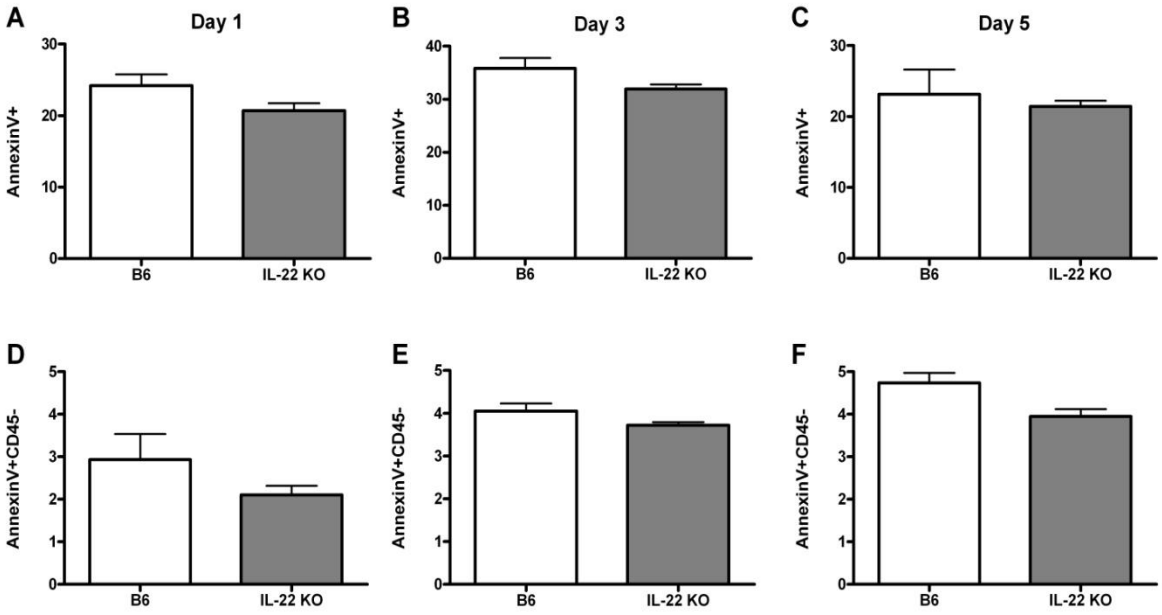

Figure 6. IL-22 is not required to prevent apoptosis in spleens during primary systemic LM infection.

B6 and IL-22 KO mice were i.v. infected with $1x10^4$ LM. Spleens were harvested 1, 3, and 5 days p.i. Percentages of apoptotic cells were determined by flow cytometry based on expression of Annexin V (A, B, C). Percentage of apoptosis in non-hematopoietic epithelial cells was determined by staining for Annexin V and CD45 (D, E, F). Two-way ANOVAs did not detect significant effects of mouse strain ($p \geq 0.05$). All data are expressed as the mean $\pm$ SEM (n = 5/group).

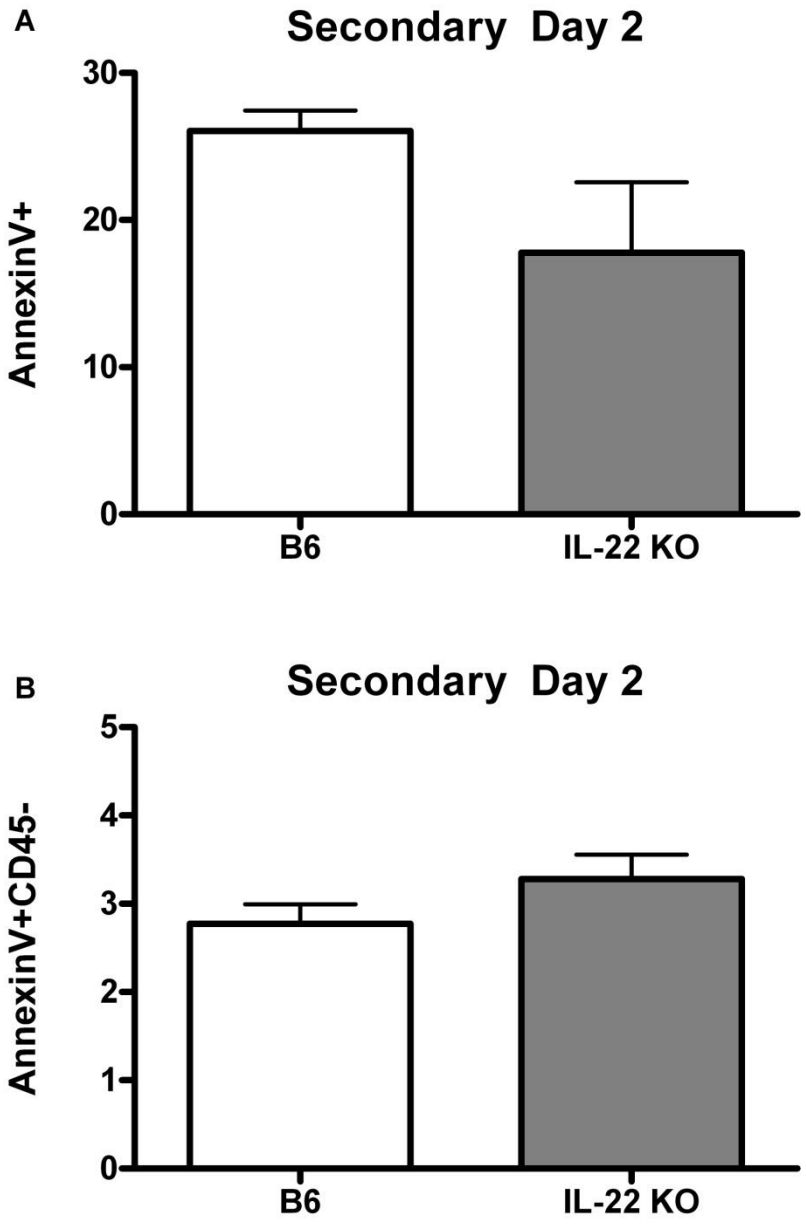

Figure 7. IL-22 is not required to prevent apoptosis in spleens during secondary systemic LM infection.

B6 and IL-22 KO were i.v. infected with $1x10^3$ LM, then re-infected six-weeks later with $1x10^6$ LM. Spleens were harvested 2 days p.i. Percentages of apoptotic cells were determined by flow cytometry based on expression of Annexin V (A). Percentage of apoptosis in non-hematopoietic epithelial cells was determined by staining for Annexin V and CD45 (B). t-tests did not detect significant effects of mouse strain ($p \geq 0.05$). All data are expressed as the mean $\pm$ SEM (n = 5/group).

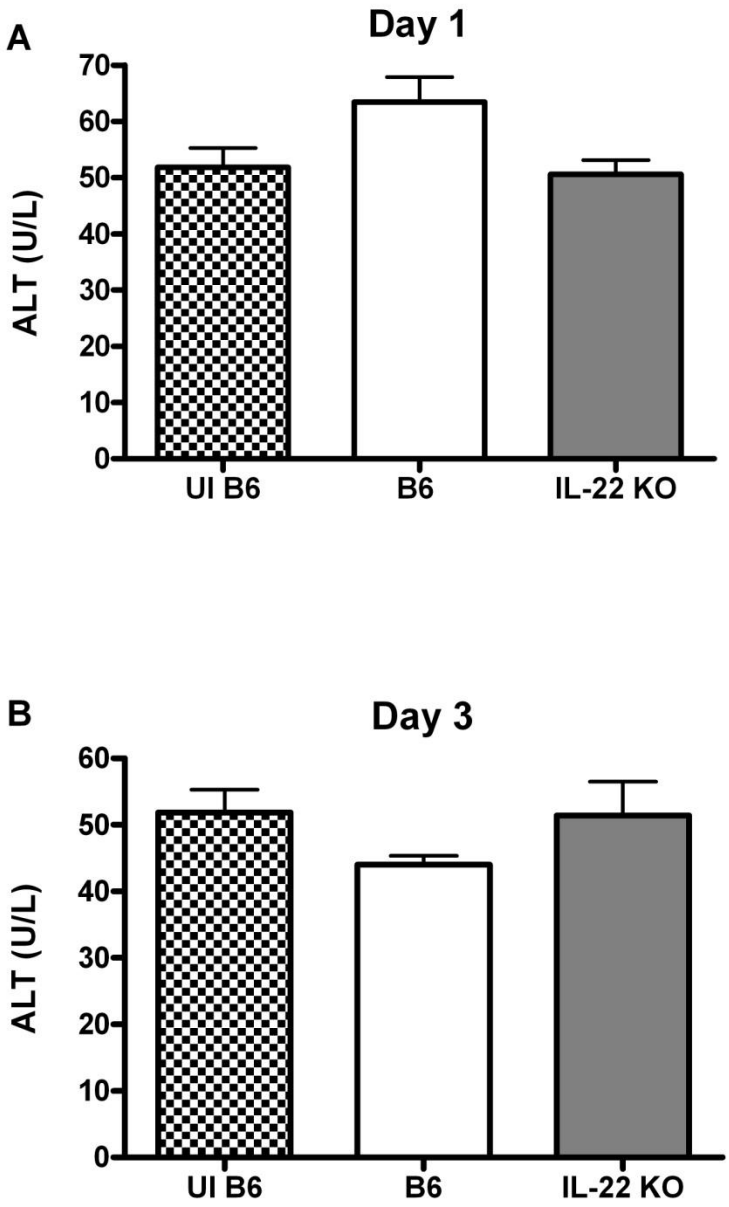

Figure 8. IL-22 is not required for tissue protection in livers during primary systemic LM infection.

B6 and IL-22 KO mice were i.v. infected with $1x10^4$ LM. Serum was harvested 1 and 3 days p.i. and from un-infected mice (UI B6) to be analyzed with an ALT detection kit. One-way ANOVAs did not detect significant effects of mouse strain ($p \geq 0.05$). All data are expressed as the mean $\pm$ SEM (n = 5/group).

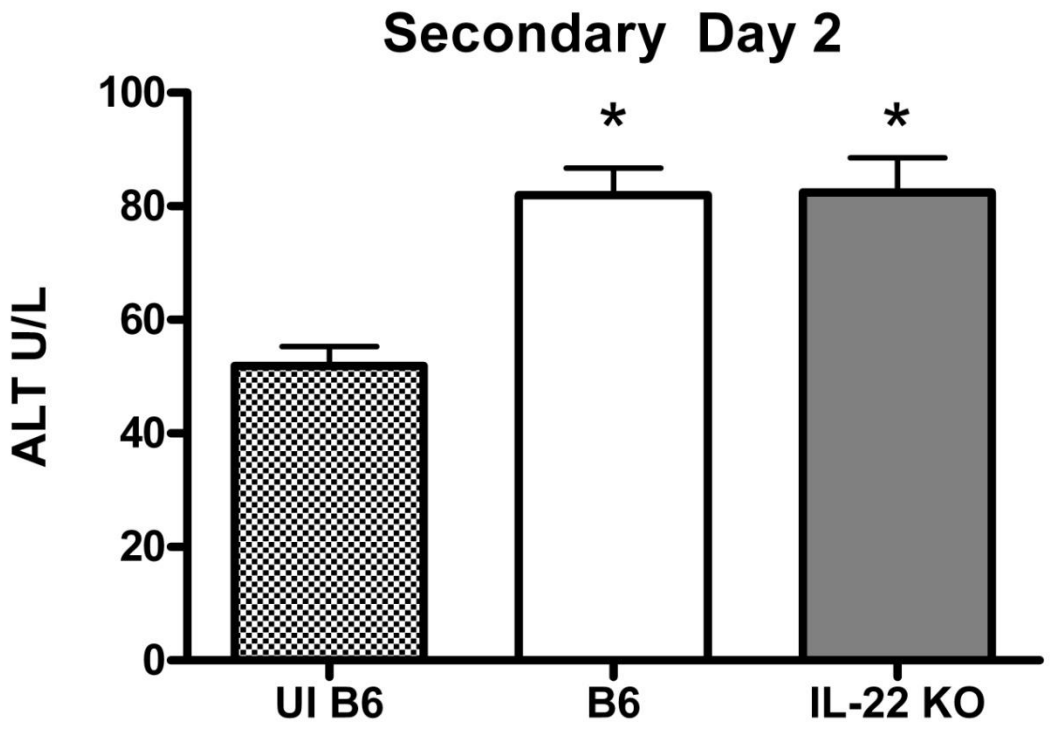

Figure 9. IL-22 is not required for tissue protection in livers during secondary systemic LM infection.

B6 and IL-22 KO mice were i.v. infected with $1x10^3$ LM, then re-infected six-weeks later with $1x10^6$ LM. Serum was harvested 2 days p.i. and from un-infected mice (UI B6) to be analyzed with an ALT detection kit. A one-way ANOVA detected a significant effect of mouse strain ($\underline{p} \leq 0.05$). An * indicates a significant difference from UI B6 ($\underline{p} \leq 0.05$). All data are expressed as the mean $\underline{+}$ SEM (n = 5/group).

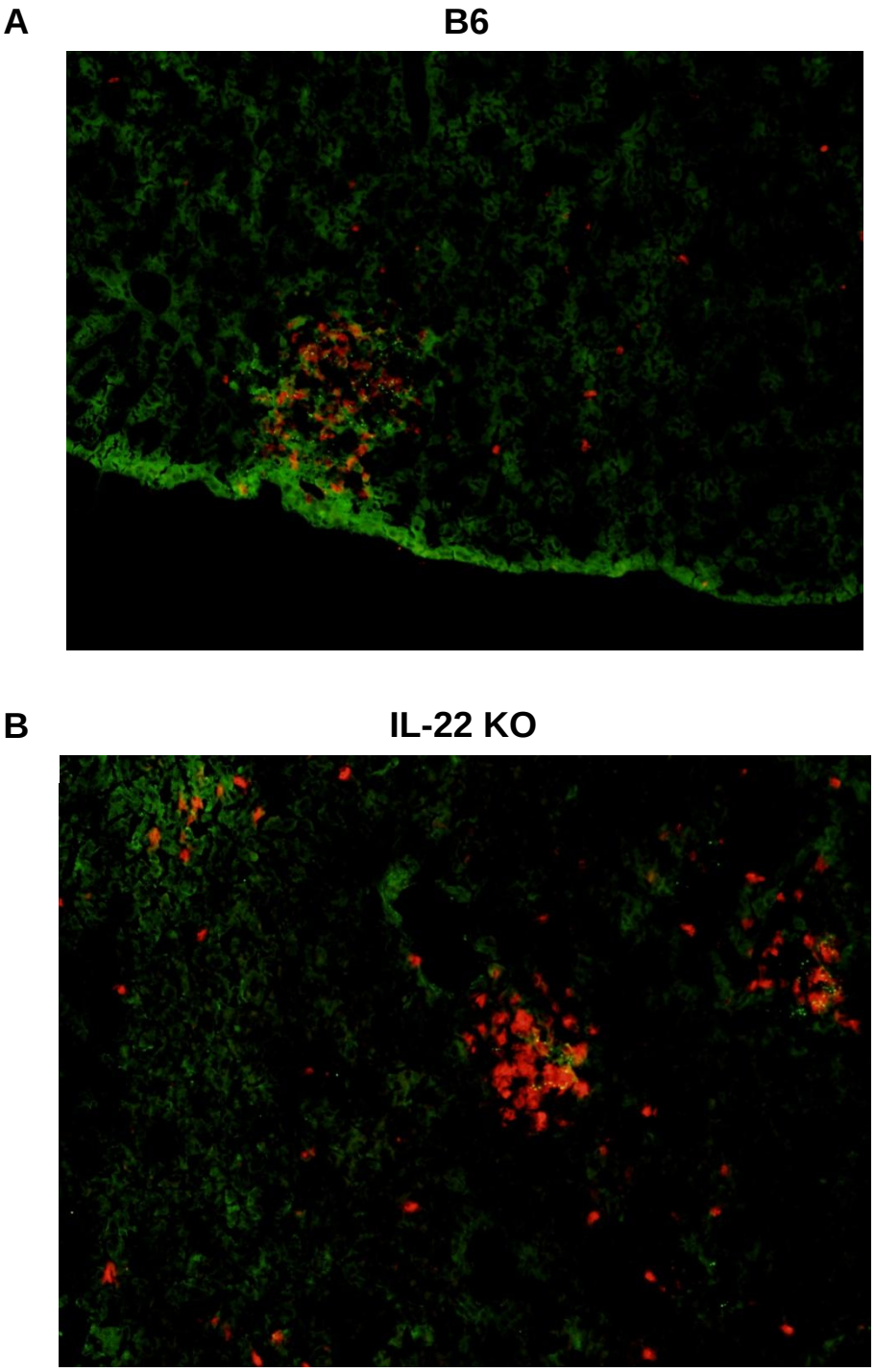

Figure 10. IL-22 is not required for the formation of LM lesions in the liver during primary systemic infection.

B6 (A) and IL-22 KO (B) mice were i.v. infected with $1x10^4$ LM. Livers were harvested at day 3 p.i. Using immunohistochemistry, 5 µm sections were stained for Ly6G+ neutrophils (red) and LM (green, yellow when merged). Total magnification was 100X.

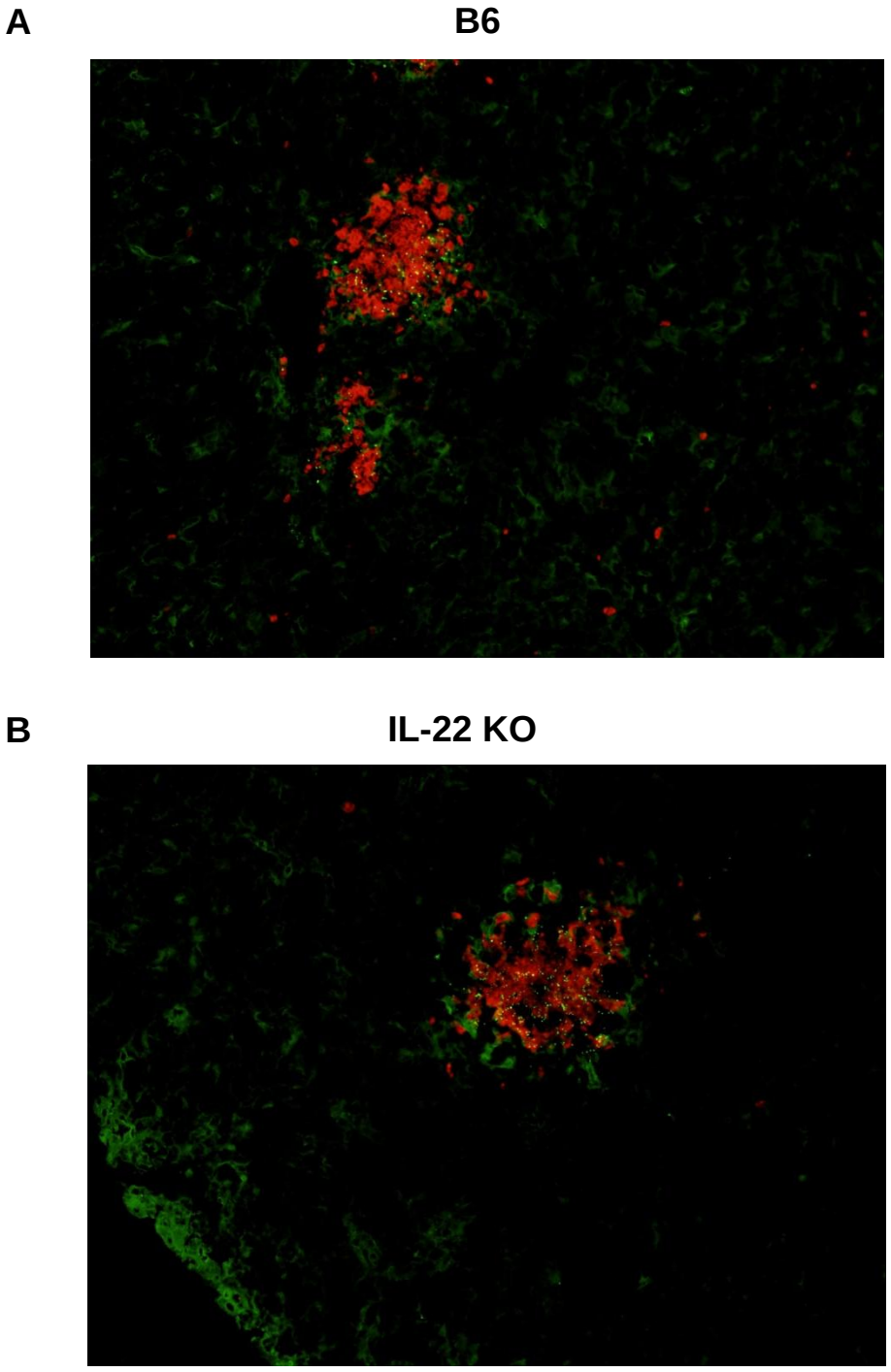

Figure 11. IL-22 is not required for the formation of LM lesions in the liver during secondary systemic infection.

B6 (A) and IL-22 KO (B) mice were i.v. infected with $1x10^3$ LM, then re-infected six-weeks later with $1x10^6$ LM. Livers were harvested at day 2 p.i. Using immunohistochemistry, 5 µm sections were stained for Ly6G+ neutrophils (red) and LM (green, yellow when merged). Total magnification was 100X.

CHAPTER IV

THE ROLE OF IL-22 DURING MUCOSAL LM INFECTION

IL-23 is required for clearance of LM during a primary mucosal i.g. infection

In a natural route of infection, LM is ingested by humans from contaminated meats and dairy products. LM moves through the intestinal epithelial layer and gets absorbed by the circulating blood before infecting the spleen and liver. To mimic the route of infection in humans, we developed an oral mucosal model of infection in mice. Because of the difference between mouse E-cadherin and human E-cadherin on intestinal epithelial cells, Internalin A on the surface of LM is not able to optimally bind to mouse E-cadherin [1]. This can be overcome by infecting mice through the mucosal i.g. route with high doses of LM [2-4]. Our lab has recently shown that IL-23 is required for survival and clearance of LM from the spleen and liver during a primary systemic LM infection [41]. To determine if IL-23 was required for survival during a mucosal i.g. LM infection, B6 and IL-23 KO mice were i.g. infected with LM and observed for 10 days. Although there were no differences in survival between B6 and IL-23 KO mice, IL-23 KO mice lost significantly more weight than B6 mice (data not shown), suggesting that IL-23 is playing a role during mucosal LM infection. To determine if IL-23 is required for bacterial clearance during a mucosal infection, B6 and IL-23 KO mice were i.g. infected and spleens and livers were harvested at days 1 and 3 p.i. to determine bacterial burden. Although this did not

reach statistical significance, IL-23 KO mice had greater LM CFUs as compared to B6 mice (**Figure 12**), suggesting that IL-23 is required for clearance of LM during a mucosal i.g. infection.

LM induces IL-22 production and IL-22 production requires IL-23 during mucosal i.g. infection

To determine if IL-22 is produced during mucosal LM infection, serum and spleens were harvested from B6 and IL-23 KO mice that were i.g. infected with LM/strep^r (strain of LM that is streptomycin resistant – see next section for explanation) for 3 days. The concentration of IL-22 was measured in the serum and splenocyte culture supernatants stimulated with or without heat-killed LM/strep^r (HKLM) or IL-23 using ELISA. In IL-23 KO mice, the amount of IL-22 was reduced in the serum and splenocyte culture supernatant with or without HKLM compared to LM infected B6 mice (**Figure 13A, 13B,** and **13C**). Therefore, LM infection induces the production of IL-22 and the production of IL-22 is dependent on IL-23 during a primary mucosal i.g. infection. However, there were no differences in IL-22 production in splenocytes re-stimulated with IL-23 (**Figure 13D**). This suggests that the cells capable of producing IL-22 are still present in IL-23 KO mice, but are not able to secrete IL-22 without the presence of IL-23.

IL-22 is not required for survival or clearance of LM during mucosal i.g. infection

IL-22 has been shown to induce the production of antimicrobial peptides from epithelial cells which can lead to clearance of bacterial infections at mucosal surfaces [32;40;44]. Because we are infecting mice via the mucosal i.g. route, the LM must pass through the intestine before disseminating to the spleen and liver, so we were also interested in observing bacterial burden in

the intestines. The intestine hosts commensal bacteria, making it very difficult to accurately measure LM CFUs in this organ. However, intestinal commensal bacteria are sensitive to streptomycin. To selectively measure LM CFUs in the intestine, we created a streptomycin resistant strain of LM. To do this, wild-type LM was plated on streptomycin plates. In order to grow, a mutation occurred, allowing LM to become streptomycin resistant. Therefore, the only bacteria able to grow on streptomycin plates are the streptomycin resistant LM (LM/strep^r). We next wanted to discern if IL-22 was required for survival during a mucosal i.g. LM infection. Mice were i.g. infected with $1x10^8$ LM/strep^r and observed for 14 days. Weights and clinical signs of illness (posture and condition of fur) of these mice were also observed. There were no differences in survival (**Figure 14**), weight, or clinical signs of illness (data not shown) between B6 and IL-22 KO mice, suggesting that IL-22 production is not required for survival during a primary mucosal i.g. infection.

Although no differences in survival between B6 and IL-22 KO mice were observed, we wanted to determine if IL-22 was required for clearance of LM from tissues during a mucosal i.g. infection. To determine the impact of IL-22 on bacterial clearance during a primary mucosal LM infection, B6 and IL-22 KO mice were i.g. infected with LM/strep^r and spleens, livers, and intestines were harvested at days 1 and 3 p.i.. There were no LM CFU differences between B6 and IL-22 KO mice at days 1 (data not shown) and 3 (**Figure 15**) p.i. However, IL-22 may have a role during a secondary mucosal infection. In order to determine if IL-22 was required for clearance of LM from a secondary mucosal i.g. LM infection, B6 and IL-22 KO mice were infected, then re-infected six weeks later and spleens, livers, intestines, and blood were harvested. Similar to the systemic i.v. model of infection, blood CFUs were measured because LM may not be able to effectively infect spleen or liver tissue if there was tissue damage present.

However, no LM/strep^r CFUs were present in the blood of either B6 or IL-22 KO mice. There were also no differences in LM/strep^r CFU counts between B6 and IL-22 KO present in the spleen, liver, and intestine during secondary mucosal i.g. infection (**Figure 16**). Therefore, during a mucosal i.g. infection, IL-22 does not play a role in clearance of LM from the spleen, liver, or intestine.

IL-22 production is dependent on IL-23 during a primary mucosal LM infection (**Figure 13**). We were interested in determining if IL-22 production was dependent on IL-23 during a secondary mucosal i.g. LM/strep^r infection. B6 and IL-23 KO mice were infected, then re-infected six weeks later. Serum and spleens were harvested at day 3 p.i. and cultured for two days. The production of IL-22 was measured with ELISA in splenocyte culture supernatants with or without HKLM or IL-23. In the serum (**Figure 17A**) and un-stimulated splenocytes (**Figure 17B**), IL-22 production depended on IL-23. However, unlike what was observed in the primary mucosal LM infection, IL-22 was produced by splenocytes from IL-23 KO mice cultured with HKLM or IL-23 and there was no difference between the two strains (**Figure 17C** and **17D**). This suggests that IL-23 is required for IL-22 production during a primary mucosal i.g. LM infection (**Figure 13A, 13B,** and **13C**) but a factor other than IL-23 was able to induce the production of IL-22 during a secondary mucosal i.g. infection.

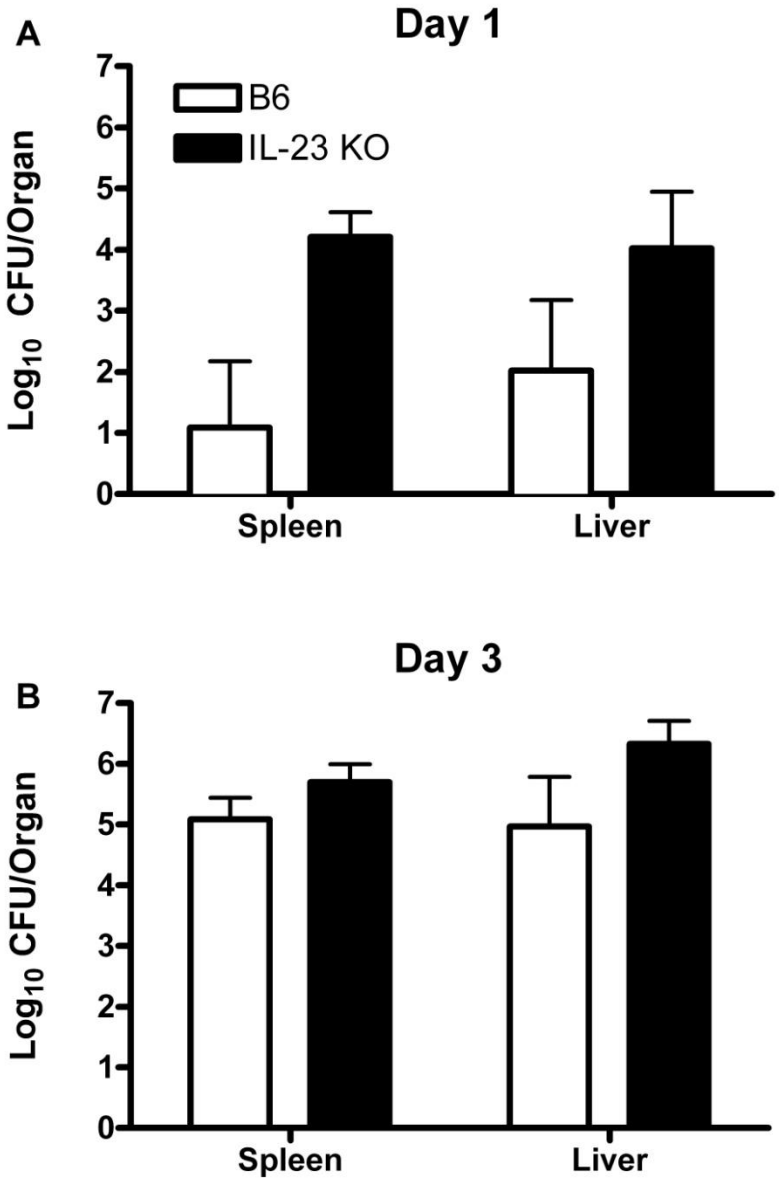

Figure 12. IL-23 appears to be required for clearance of LM from the spleen and liver during a primary mucosal LM infection.

B6 and IL-23 KO mice were i.g. infected with $1x10^7$ LM. Spleens and livers were harvested at days 1 (A) and 3 (B) p.i. and bacterial burdens were determined. Two-way ANOVAs did not detect significant effects of mouse strain ($\underline{p} \geq 0.05$). These data are representative of two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 3-4/group).

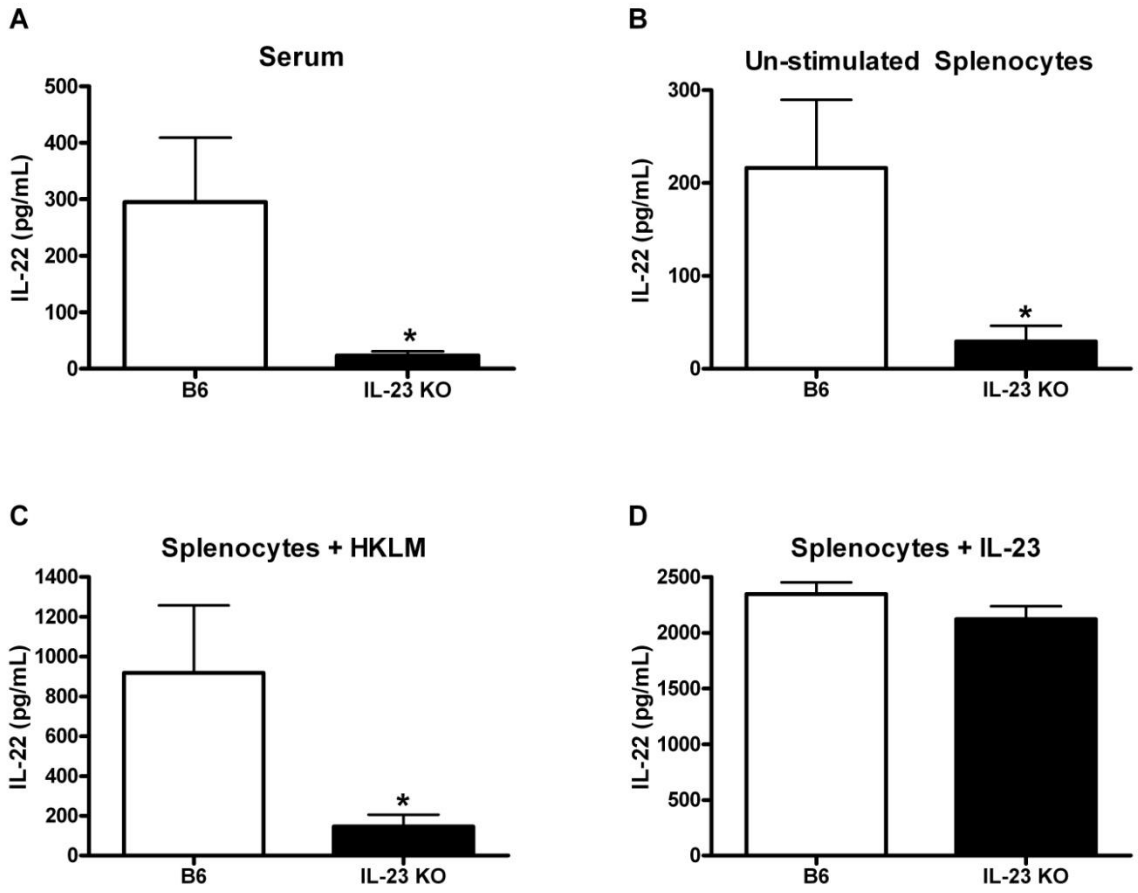

Figure 13. IL-22 is induced during mucosal LM infection and requires IL-23 during a primary mucosal infection.

Serum and spleens were harvested from uninfected B6 mice (UI B6) and B6 and IL-23 KO mice i.g. infected with $1x10^7$ LM/strep^r for 3 days. IL-22 concentration was measured using ELISA in serum (A) and splenocyte culture supernatants from un-stimulated (B), HKLM (C), or IL-23 (D) stimulated cultures. Two-way ANOVAs detected significant effects of mouse strain ($p \leq 0.05$). An * indicates a significant difference from B6 ($p \leq 0.05$). These data are representative of two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 4/group).

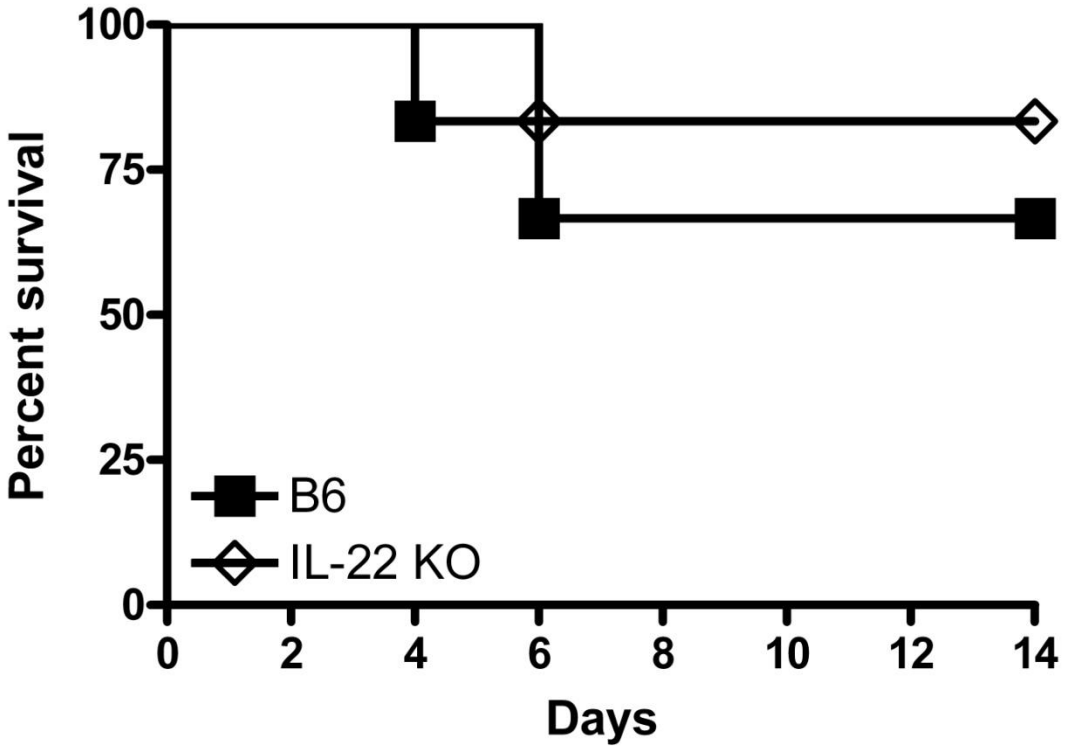

Figure 14. IL-22 is not required for survival during mucosal LM infection.

B6 and IL-22 KO mice were i.g. infected with $1x10^8$ LM/strep^r for a survival study. A log rank analysis did not detect a significant difference between the survival curves of B6 and IL-22 KO mice, ($\underline{p} \geq 0.05$), (n=4/group).

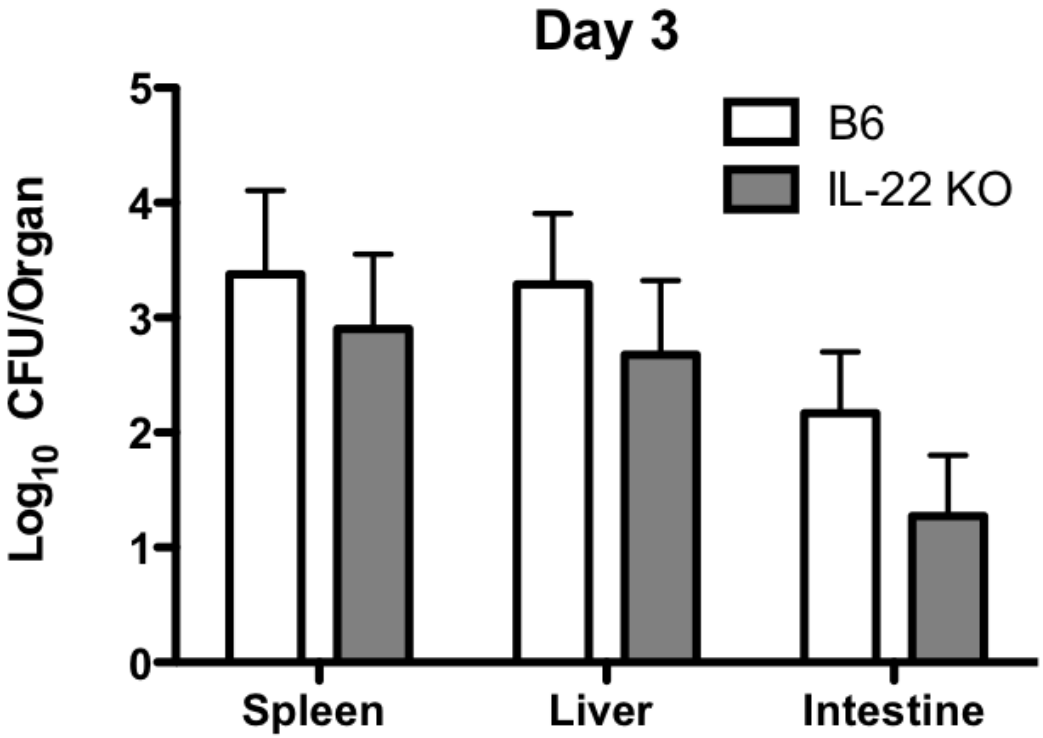

Figure 15. IL-22 is not required for clearance of bacteria from the spleen, liver, and intestine during a primary mucosal LM infection.

B6 and IL-22 KO mice were i.g. infected with $1x10^7$ LM/strep^r. Spleens, livers, and intestines were harvested day 3 p.i. and bacterial burdens were determined. A two-way ANOVA did not detect significant effects of mouse strain ($p \geq 0.05$). These data are combined from two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 12/group).

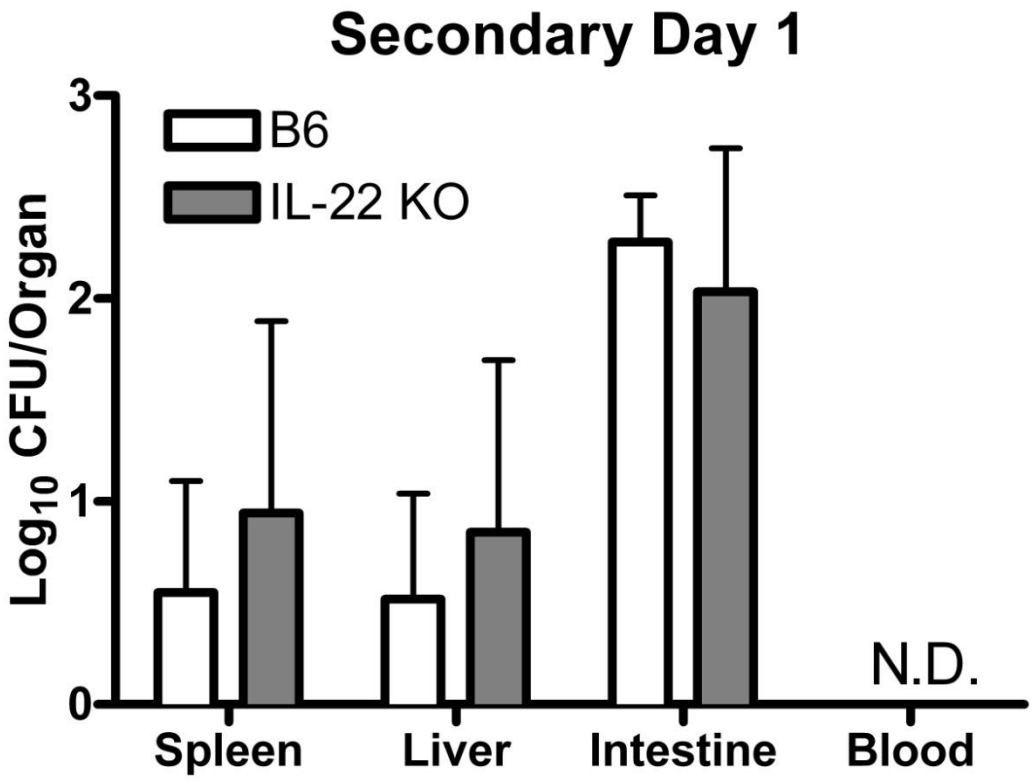

Figure 16. IL-22 is not required for clearance of bacteria from the spleen, liver, intestine and blood during a secondary mucosal LM infection.

B6 and IL-22 KO mice were i.g. infected with $1x10^7$ LM/strep^r, then re-infected six-weeks later with $1x10^8$ LM/strep^r. Spleens, livers, intestines, and blood were harvested at day 1 p.i. and bacterial burdens were determined. A two-way ANOVA did not detect significant effects of mouse strain ($p \geq 0.05$). These data are representative of two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 4/group). N.D., No Data.

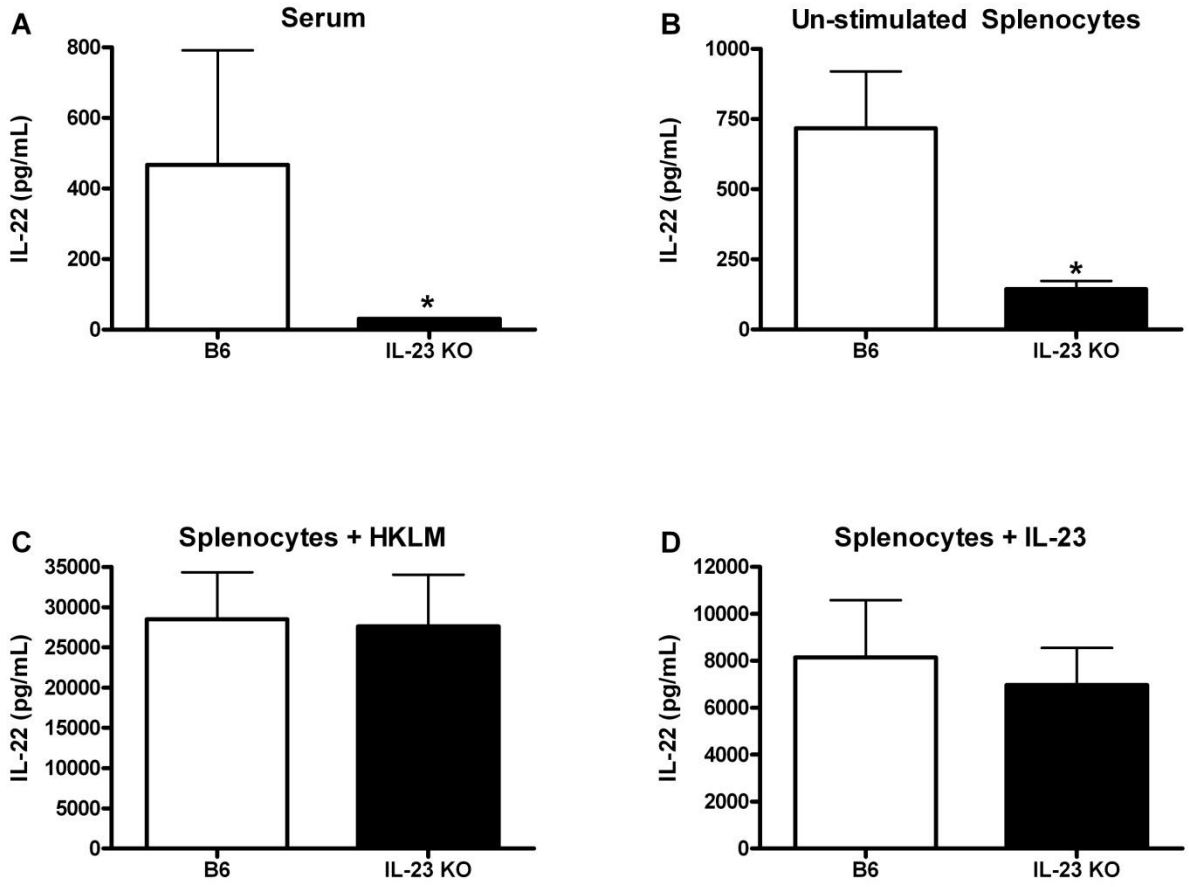

Figure 17. A factor other than IL-23 is able to induce IL-22 production when splenocytes are stimulated with HKLM.

Serum and spleens were harvested from B6 and IL-23 KO mice i.v. infected with $1x10^7$ LM/strep^r. Mice were re-infected with $1x10^8$ LM/strep^r six weeks later for a secondary infection. IL-22 concentration was measured using ELISA in serum (A) and splenocyte culture supernatants from un-stimulated (B), HKLM (C), or IL-23 (D) stimulated cultures. t-tests detected significant effects of mouse strain ($p \leq 0.05$). An * indicates a significant difference from B6 ($p \leq 0.05$). These data are representative of two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 4/group).

CHAPTER V

DISCUSSION

IL-23 is a cytokine that is required for clearance of bacteria from the spleen and liver during both a systemic and mucosal LM infection (**Figure 12**) [41]. To date, the primary role discovered for IL-23 is to maintain lymphocytes that secrete IL-17A, IL-17F, and IL-22 or to directly induce IL-22 [24;40]. The IL-23/IL-17 axis has been shown to have the ability to optimally recruit neutrophils to the liver during a primary systemic i.v. LM infection[41]. These IL-17 recruited neutrophils may be playing a role in the clearance of LM from the liver (Meeks, et al, manuscript in preparation). However, the IL-23 dependent mechanism of bacterial clearance from the spleen is unknown. IL-22 is another downstream cytokine of IL-23 and has been previously reported to be able to induce the secretion of antimicrobial peptides [24;40] as well as protect tissues from damage by preventing apoptosis [44;52]. Both functions of IL-22 are potential mechanisms by which this novel cytokine may decrease susceptibility of LM infected mice.

LM has the ability to induce the production of IL-22 during a primary infection (**Figure 1** and **Figure 13**). During both a primary systemic and primary mucosal infection, the production of IL-22 during a LM infection requires IL-23 (**Figure 1** and **Figure 13**). This IL-23 dependent optimal production of IL-22 has also been seen in other infectious models. IL-22 production is induced in mice during *Salmonella enterica* [33], *Toxoplasma gondii* [66], as well as in an induced

model of colitis [67]. When IL-23 was added back into splenocyte cultures, the production of IL-22 was restored, suggesting that the cells that are capable of producing IL-22 are present in IL-23 KO mice, but IL-22 is not being secreted (**Figure 1D** and **Figure 13D**). However, although IL-23 is required for the optimal production of IL-22, in the absence of IL-23, LM can induce the production of IL-22 at lower amounts (**Figure 1** and **Figure 13**). This suggests IL-22 is not completely dependent on IL-23 for production, and another factor is able to induce the production of IL-22. During secondary LM infection, IL-22 production is not regulated by IL-23. In the absence of IL-23, IL-22 is still able to be produced, and at comparable levels to B6 mice, suggesting that during a secondary LM infection IL-23 is not required to induce IL-22 production (**Figure 5** and **Figure 17**). Others have proposed that IL-6 is sufficient to induce the production of IL-22 [61;63;68]. Conflicting literature, however, suggest that IL-6 is not required for IL-22 induction during ConA mediated hepatitis [52]. To test if IL-6 or a combination of IL-6 and IL-23 are sufficient for IL-22 production during LM infection, these cytokines can be added to a B6 splenocyte culture and IL-22 secretion can be measured. To determine if IL-6 is necessary for IL-22 production, IL-6 can be neutralized in a splenocyte culture. It has also been suggested that IL-12 can induce the production of IL-22. Naïve T cells cultured with either IL-12 or IL-23 were able to produce IL-22 [69]. The presence of IL-6 or IL-12 might be inducing the production of IL-22 in the absence of IL-23, suggesting that IL-22 is not exclusively associated with the IL-23/IL-17 axis [70].

LM can induce the production of antimicrobial peptides such as RegIIIγ, and these antimicrobial peptides are required for clearance of LM [6;48]. IL-22 has been shown to have the ability to induce the production of antimicrobial peptides [32;40;43-46;56;57;71] and might be regulating the antimicrobial peptide production during LM infection. In vitro, LM is susceptible to some

antimicrobial peptides including RegIIIγ, protegrins, ubiquicidin, cryptidins (α-defensins), and cathelin-related antimicrobial peptides (CRAMP) [47-51], but it has not been determined if IL-22 dependent production of antimicrobial peptides can aid in the clearance of LM during infection. However, there were no differences in bacterial clearance between B6 and IL-22 KO mice during primary systemic or primary mucosal LM infections (**Figure 3** and **Figure 15**). IL-22 is also not required for LM clearance in a systemic i.v. or mucosal i.g. secondary infection (**Figure 4** and **Figure 16**). This suggests that any IL-22 induced production of antimicrobial peptides is not required for bacterial clearance during an LM infection. Congruent with this, Zenewicz et al saw no differences in LM burdens between B6 and IL-22 KO mice day 3 post infection [52]. Other labs have seen similar results in other infectious models, including parasite infection with *T. gondii* and *Schistosoma mansoni*, bacterial infections with *Mycobacterium avium* and *Mycobacterium tuberculosis* [70], as well as fungal infections with *Candida albicans* [38]. Production of IL-22 does not appear to be the mechanism of IL-23 clearance of LM from the liver, supporting the hypothesis that the IL-23/IL-17 axis recruitment of neutrophils aids in LM resistance in the liver [41]. The mechanism of IL-23 clearance of LM from the spleen remains unknown since IL-22 is not required for LM clearance.

Although there were no differences in bacterial burdens between B6 and IL-22 KO mice during the mucosal i.g. LM infection, inconsistencies have been observed in LM bacterial burdens during mucosal infections, leading to difficulties obtaining reproducible results [2-4;72]. There could be many explanations for this lack of consistent CFUs. During the oral mucosal route of infection, LM must pass through the intestinal epithelial barrier before it can disseminate to the spleen and liver. In humans, Internalin A on the LM surface binds with human epithelial cadherin which allows it to be phagocytized by the intestinal epithelial cells. Because of the

single amino acid change in mouse E-cadherin, LM is not able to optimally bind to the intestinal epithelial cells, therefore not allowing a consistent number of LM cross the epithelial border to infect the spleen and the liver [1]. In order to use a mouse model for mucosal LM infections, a transgenic mouse model expressing human E-cadherin is available [4]. The intestine is also colonized with commensal bacteria that compete with invading bacteria for nutrients, and which also prevent invading bacteria from entering the intestinal barrier [70;73-78]. LM may not be able to out-compete the commensal bacteria and is therefore unable to enter the bloodstream to infect the spleen and liver. High variability in the bacterial load seen in the spleen and liver can be avoided by infecting mice systemically with LM. Injection of LM directly into the bloodstream allows LM to bypass the intestine and disseminate straight to the spleen and liver [4]. In fact, similar results were seen in the systemic i.v. and mucosal i.g. models of infection, indicating that that the systemic i.v. route of infection is an appropriate infectious model for the natural route of LM infection. Younger, smaller mice can also be used, as these mice do not yet have the established commensal bacteria, therefore making them more susceptible to LM infection[3;74;79]. Germ-free mice that do not have intestinal commensal bacteria are another possible solution to this problem [73;75-77]. However, these germ-free mice are more susceptible to infection and are not representative of the natural route of LM infection in humans.

IL-22 has previously been shown to protect tissues against damage during infection with *Klebsiella pneumoniae* or ConA stimulation [44;52]. However, we have found that IL-22 is not required for spleen or liver protection during primary or secondary systemic LM infection (**Figures 6-11**). This was also seen in the livers of mice infected with *S. mansoni* and *T. gondii*, and in the lungs of mice infected with *M. tuberculosis* and *M. avium* [70]. As mentioned previously, the natural human route of infection for LM is an oral mucosal infection. IL-22 might

be playing a role in protecting the intestinal tissue during a mucosal LM infection. During an oral *T. gondii* infection, the intestine of WT mice had more pathology than the intestine of wild type mice treated with anti-IL-22 despite the fact that parasite burdens were the same [70]. Even though there were no differences in LM clearance between B6 and IL-22 KO mice during a mucosal oral infection, there might be intestinal tissue damage. To determine if the intestinal epithelial layer is damaged, immunohistochemistry can be performed to observe the tissue structure of the intestine.

IL-22 is produced during LM infection and is regulated by IL-23 during a primary infection but the function of IL-22 during LM infection remains unknown. In other infectious models, the known functions of IL-22 are the ability to induce the production of antimicrobial peptides and protection of tissues. However, mice lacking IL-22 did not have any differences in the ability to clear LM or protection of tissues compared to B6 mice during LM infection. In a recent publication, there were no differences in worm or egg burden of *S. mansoni* of IL-22 KO mice or wild type mice that had IL-22 neutralized, when compared to control mice [70]. IL-22 was also found to be dispensable for bacterial clearance in gram-positive mycobacterium infections such as *M. avium* and *M. tuberculosis* [70]. In order to determine the function of IL-22 during LM infection, B6 and IL-22 KO mice can be infected and a microarray can be performed. This would determine which genes are up-regulated or down-regulated during LM infection in IL-22 KO mice compared to B6 mice, shedding some light on the role of IL-22.

The source of IL-22 during LM infection is currently unknown. Literature suggest that lymphoid tissue inducer (LTi) cells and NKp46+ cells are capable of producing IL-22 [78;80]. Human LTi cells and mouse LTi-like cells are involved in the formation of secondary lymphoid tissue such as lymph nodes, spleen, tonsils, and intestinal Peyers patches [80;81]. These cells can

secrete IL-22 in response to IL-23 as well as PMA and ionomycin [82;83]. It has been suggested that mouse LTi-like cells differentiate into NK cells [84]. NKp46+ cells have been implicated in the production of IL-22 in the intestine [85;86]. Both LTi cells and NKp46+ cells require RORγt transcription factor for the production of IL-22 [85;86]. IL-23 and intestinal commensal bacteria can signal NKp46+ cells to induce the secretion of IL-22 [85;87-89]. During a natural, mucosal route of LM infection, these cell types might be producing IL-22 in response to signals from intestinal commensal bacteria or signals from LM induced cytokines.

We have found that IL-22 is not required for clearance of LM from a primary or secondary infection. In order to determine whether IL-22 was playing a role in a more natural route of LM infection, a mucosal i.g. model was established. Although variability was present in this route of infection, IL-22 does not appear to be required for clearance of LM. This similar result to the systemic i.v. route of infection suggests that the systemic i.v. route of LM infection is a good model to study the effects of LM. We have also established that IL-22 does not play a role in protecting the spleen and liver from damage during a primary or secondary LM infection. While IL-22 is produced during LM infection and this production is regulated by IL-23, the function of IL-22 currently remains unknown during a LM infection.

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