Roles of Innate Lymphoid Cells on *Mycobacterium avium* infection

by
Jay Bickell

A THESIS

submitted to

Oregon State University

Honors College

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Honors Baccalaureate of Science in Biology (Honors Associate)

Presented February 27, 2020
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Abstract approved:_____
Luiz Bermudez

Mycobacterium avium subsp. *hominissuis* (MAH) is a common environmental bacterium that causes infections in immunocompromised patients such as those with HIV/AIDS, or patients with chronic lung disease such as Cystic Fibrosis. There are many strains of MAH with varying levels of virulence. Infection with MAH strains 100 and 104 have been associated with different immune responses in mice. While MAH 100 infection tends to be cleared from mice, MAH 104 has a greater virulence and grows in host tissue. What is currently unknown are the mechanisms related to this difference in host defense and virulence. Our hypothesis is that differences in submucosa innate lymphocytes response are associated with increased protection from infection. Innate Lymphoid Cells (ILC) are lymphoid cells with an important role in regulation of innate immune systems. ILCs can be categorized into three subpopulations ILC 1, ILC 2, and ILC 3 based on their cytokine production. Investigation was carried out on how macrophage anti-MAH response changes depending on activation by primary mouse lymphocytes belonging to different ILC subpopulations. Our results do not affirm the role of any one ILC subpopulation in macrophage anti-*M. avium* ability. Our findings instead support the conclusion that mycobacterium infected macrophages suppress the stimulatory function of ILCs.

Key Words: *Mycobacterium avium* subsp. *hominissuis*¸ Innate Lymphoid Cells, macrophage, MAH, Interferon-gamma, IL-4, Il-12, Il-17, IL-22, IL-33, mouse, spleen.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Introduction

Mycobacterium avium subs. *homnissisus* (MAH) is an opportunistic pathogen that exists ubiquitously in the environment[1]. It is associated with disease in immunocompromised patients and individuals with chronic lung disease such as Cystic Fibrosis[1]. Present in environmental soil and water, as well as household sources such as shower heads and pools, MAH is acquired via inhalation or ingestion[1,3]. Infection is difficult to treat, requiring lengthy courses of multiple antibiotics, and therefore presents a significant public health issue[1,20].

Following ingestion or inhalation, and subsequent crossing of intestinal or respiratory mucosa, MAH infects macrophages. From here the bacterium is capable of altering the host cell in order to increase survival; for example, one strategy employed by MAH is to prevent acidification of macrophage phagosomes[2]. Bacteria can then replicate intracellularly before disseminating throughout the body[2,21].

Host immune response to MAH infections are not fully understood. Many strains of MAH have varying levels of virulence. Infection with MAH strains 100 and 104, for example, have been associated with different immune responses in mice. While MAH 100 infection tends to be cleared from mice, MAH 104 has a greater virulence and grows in host tissue[3]. What is currently unknown are the mechanisms which would explain this difference in virulence.

The aim of this work was to examine whether these differences in virulence are partially due to the roles of Innate Lymphoid Cells in the host immune response to MAH. Innate Lymphoid Cells (ILCs) are a newly described cell population, but increasingly relevant players in the complex field of the immune system[4]. ILCs can be thought of as the bridge that gaps innate and adaptive immunity, as they respond to cellular stimulation with the release of cytokines to drive immune responses[4]. Specifically, ILCs are found in abundance within mucosal

surfaces, highlighting their potential importance as a line of defense against infectious disease[5,6]. Furthermore, ILCs can be categorized into three subpopulations based on their differing functions. All three ILC subpopulations can be encountered in lungs of healthy humans and are very likely stimulated upon contact with infectious agents[13]. MAH bacteremia also have been shown to play a role in modulating the presence of these ILC subpopulations during and after *Mycobacterium tuberculosis* infection, affirming the significance of these cells during infection[6].

The three different subpopulations of ILCs are defined by the first order cytokines which differentiate them, and then the second order cytokines which are secreted in response to challenge infection[7] (Diagram 1). ILC1 cells are activated by interleukin (IL)-12 leading to the production of interferon gamma (IFNγ), and have similar function to type 1 helper T cells playing an important and multi-faceted role in responding to intracellular pathogens[7]. Additionally, ILC-1 functions resemble Natural Killer (NK) cells in many aspects, such as being capable of stimulating infected macrophages to fight the infection[4,9]. IFNγ released by ILC1 acts on macrophages to control mycobacterium infections by activating their antimicrobial properties[8].

ILC-2 cells are activated by IL-33 and secrete IL-4 having a similar role to type 2 helper T cells. These cells are encountered in lungs at higher levels than other ILC subpopulations before infections arise, and play a role in surveillance of airway epithelium and maintenance of the epithelium when damaged[7]. Raised levels of IL-4 are detected in MAH infections, however their role in in T-cell regulation are not fully defined[10]. Evidence in *M. tuberculosis* infections suggests they have an early effect in T-cell regulation, macrophage activation, and inflammation that determine whether the infection becomes latent or progressive[10].

ILC-3 cells are stimulated by IL-23 and produce IL-17 similar to their counterpart TH17 cells[7]. ILC-3 has been shown to have an early protective role in mycobacterium infections, which

leads to rapid accumulation of ILC-3 in lung tissues which coincides with accumulation of alveolar macrophages[7,11]. Overall this ILC subpopulation has the most diverse roles of the ILC subpopulations and these roles are shaped by environmental conditions in the body[11].

Investigation was carried out on how macrophages responded to activation by primary mouse lymphocytes belonging to the three Innate Lymphoid Cell subpopulations. It is important to note that in this experimental model mouse lymphocytes were harvested from the spleen. This was done in part due to the relative ease of harvesting circulatory lymphocytes rather than mucosal ones. While the lungs can directly contact respiratory pathogens, the spleen is a lymphatic organ that is in contact with the circulatory system to stimulate or suppress immune responses[12]. ILCs replenish and self-maintain locally leading to distinct microenvironments of these cells in each organ that been shown to cause phenotypic and functional differences of lymphocytes[7]. In both lung and spleen environments' lymphocytes are regulated by the surrounding cells. For example, alveolar macrophages inhibit NK activity while spleen macrophages do not, but are able to further prime NK cytotoxicity and proliferation[12]. Additionally, in the spleen lymphocytes can be exposed to phagocytic and endothelial cells[12]. Whereas in the lungs epithelial cells secrete cytokines upon contact with pathogens for cell differentiation, in the circulation differentiation relies on cytokines produced by phagocytic cells, circulating lymphocytes, and endothelial cells[12]. Consideration of these microenvironment differences and their relevance to results will be further discussed in this paper.

By infecting primary mouse spleen lymphocytes to obtain supernatant representing differentiated ILC sub populations, we intended to determine whether ILC derived supernatant would have any role in the stimulation of macrophages, with the ability to suppress MAH infection. Specifically, it was hypothesized that one of the three ILCs populations play a

significant role in the immune response to MAH and activate macrophage's ability to inhibit MAH infection.

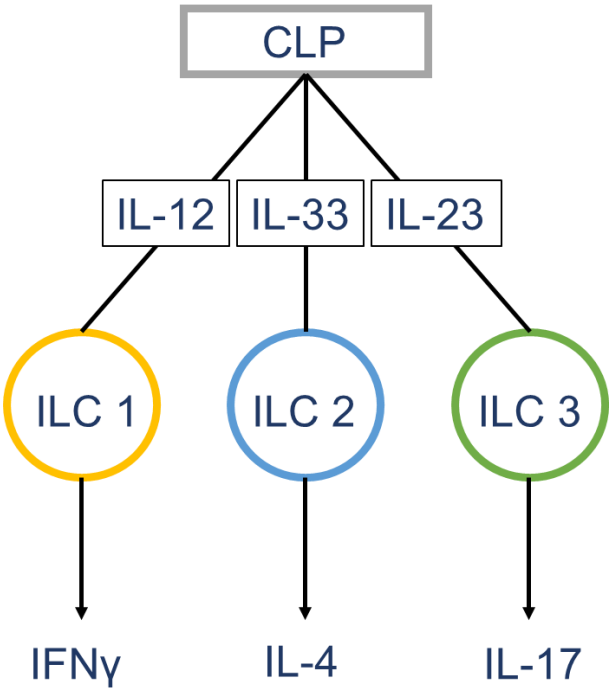

Diagram 1: Flow chart of primary cytokines (IL-12, IL-33, Il-23) that differentiate common lymphoid progenitor (CLP) into ILC Subpopulations and the cytokines produced by each subpopulation (IFNγ, IL-4, IL-3)

Materials and Methods

Bacterial strains

Two strains of *Mycobacterium avium* (MAH) 100 and 104 were used. MAH strain 104, originally isolated from the blood of an AIDS patient causes disseminated and pulmonary infection in mice while and MAH 100, also isolated from the blood of AIDS patient, is attenuated in mice[3]. Both strains were grown on Middlebrook 7H10 agar supplemented with 10% w/v oleic acid-albumin-dextrose-catalase (OADC; Hardy Diagnostics; Santa Maria, Ca) and used for experiments between 7-14 days of growth and 1-5 passages in vitro. All strains were grown at 37°C. Inoculums for all experiments were prepared in Hanks Balanced Salt Solution (HBSS, Cellgro, Manassas, VA) and syringe passaged for dispersion of the suspension before establishing the inoculum using a spectrophotometer Appropriate solution was then used to make a multiplicity of infection (MOI) of 1.

Host Cells

Peritoneal murine macrophage RAW 264.7 cell line obtained from the American Type Culture Collection (ATCC; Manassas, VA) were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI; Corning) supplemented with 10% fetal bovine serum (FBSl corning). Cells for infection assays were counted with a hemocytometer and seeded at 60% confluency in 48 well plates. Monolayers were infected 24 hours later when confluency reached approximately 80%. Because RAW cells continue to replicate after infection so 100µL of fresh media was added every 48 hours to support cell growth.

Supernatant Preparation

Mouse spleen were harvested from C57BL/6 mouse and dissected in RPMI using two sterile 22-gauge needles until there were no visible tissue. Media containing cells was then transferred into tissue culture plate and incubated at 37°C for 45 minutes. Once macrophages had adhered to the plastic, remaining lymphocytes and media were removed, and placed in fresh plates. Macrophages were then washed with HBSS and medium was replenished with fresh RPMI+10% FBS. All cells were given 24 hours to rest before infecting with MAH 104 and stimulating the lymphocyte population triggering differentiation into ILC subpopulations to create different supernatant conditions. ILC subpopulations 1, 2, and 3 were differentiated with the addition 10ng/mL of either IL-12, Il-33, or IL-23, respectively, to wells containing either lymphocytes or lymphocytes and macrophages . Controls included wells with nonstimulated cells as well as wells with heat-killed MAH 104. Supernatants were then collected directly 4, 24, and 48 hours post infection. Resulting supernatant of each well was obtained and syringe filtered before storage at -20°C for use in future assays. See appendix 1 for a guide to supernatant conditions.

Survival Assays

For each assay monolayers of RAW 264.7 cells at 60% confluency were seeded and given 24 hours to adhere to wells before infected with either MAH 100 or 104 for one hour at a MOI of 1. Inoculums were standardized between experiments using optical density measurements and confirmed through dilution, plating, and incubation at 37°C for 7-10 days in order to count viable colonies. HBSS was then used to rinse monolayers and remove remaining extra cellular bacteria. Different treatments were then added to monolayers infected with either MAH 100 or 104:

1) Supernatant Treatment*:* Post inoculation 100μL of each of the previously described supernatants was added onto separate wells in duplicate each for well infected with MAH 100 and 104. Supernatant was unfrozen, brought up to 37°C, and vortex agitated before addition to wells. Controls wells were incubated with supernatant from heat killed bacteria and additional controls wells were incubated with RPMI+ 10% FBS and absent of supernatant.

2) Cytokine Treatment: Post inoculation, 1μL of 10ng/mL of IL-4, IL-17, or IFNγ was added to monolayers infected MAH 100 or 104. Control wells were incubated in RPMI+10% FBS with no added cytokines. Each assay was done in triplicate.

3) Supernatant and Cytokine Treatment: Post inoculation, 100μL of ILC-1 differentiated supernatant and 1μL IFNγ (10 ng/mL) were added in duplicate to infected MAH 104 monolayers. Supernatant was unfrozen, brought up to 37°C, and vortex agitated before addition to wells. Controls included supernatant with heat killed bacteria and wells incubated in RPMI+10%FBS only with no IFNγ.

For all three of the above described assays, at day 2 and 4 post infection cells were lysed in 0.1% Triton-X100 (Sigma Aldrich, St. Louis, MO) for 10 minutes followed by pipetting in and out of well. Contents of wells were then serially diluted and plated onto Middlebrook 7H10 agar to quantify the number of viable bacteria present in infection. Plates were incubated at 37°C for 7-10 days until visible colonies formed for counting.

Statistical Analysis

All results are representative of either duplicate or triplicate replicates as indicated. Significance and standard deviation were calculated for all the assays. Graph Pad Prism and Excel were used for all statistical analysis. Comparison between treatment groups and controls were determined using a two tailed t-test and confirmed with a 2-way ANOVA. Significant values had $p<0.05$. All graphs were created using GraphPad Prism and tables and figures were created using Microsoft Suite.

RESULTS

Macrophage infection in presence of supernatant

To determine whether the supernatant of the three ILC subpopulations induced the ability to protect macrophage against *M. avium* infection, CFUs were determined from a survival assay. Phagocytic RAW macrophage monolayers were infected with either MAH 100 or 104 and then exposed to previously collected supernatants (refer to Diagram 2 for coded names of supernatant conditions used below).

Monolayers infected with MAH 100 showed no significant differences between control groups (supernatants of undifferentiated lymphocytes) and any of the ILC groups from the C_{100} conditions (Figure 1, panel C). This trend remained similar for ILC groups from D_{100} conditions with the exception of IL-23 differentiated lymphocytes showing significantly higher CFUs than macrophage and lymphocyte control supernatant (Figure 1, panel D).

Monolayers infected with MAH 104 showed no significant differences between control groups (supernatants of undifferentiated lymphocytes) and any of the ILC differentiated groups from the A_{104}, B_{104}, C_{104} or the D_{104} conditions (Figure 2, panel C and D). However for each ILC subpopulation in A_{104} there was significantly lower CFUs than the corresponding ILC sup

population activated from C_{104} wells. For example, A_{104} wells containing supernatant derived from lymphocytes activated with IL-12 showed a 56% decrease in CFUs compared to C_{104} wells containing supernatant derived from both lymphocytes and macrophages activated with IL-12. This trend was also seen between B_{104} and D_{104} ILC subpopulations but not statistically significant.

In general, the results show that there is not a great effect on macrophage activation when comparing different ILC subpopulations for either MAH 100 or 104 infections. Instead, the results show significant decreases of MAH 104 CFUs in ILC subpopulations derived from lymphocytes only, rather than lymphocyte and macrophage combination. The results were unexpected and raised the question of whether the expected ILC produced cytokines were present in the supernatant and if they could have an impact with direct stimulation rather than indirect introduction through activation into ILC subpopulations. The next step was to assess this by establishing a baseline of direct addition of ILC produced cytokines to macrophages.

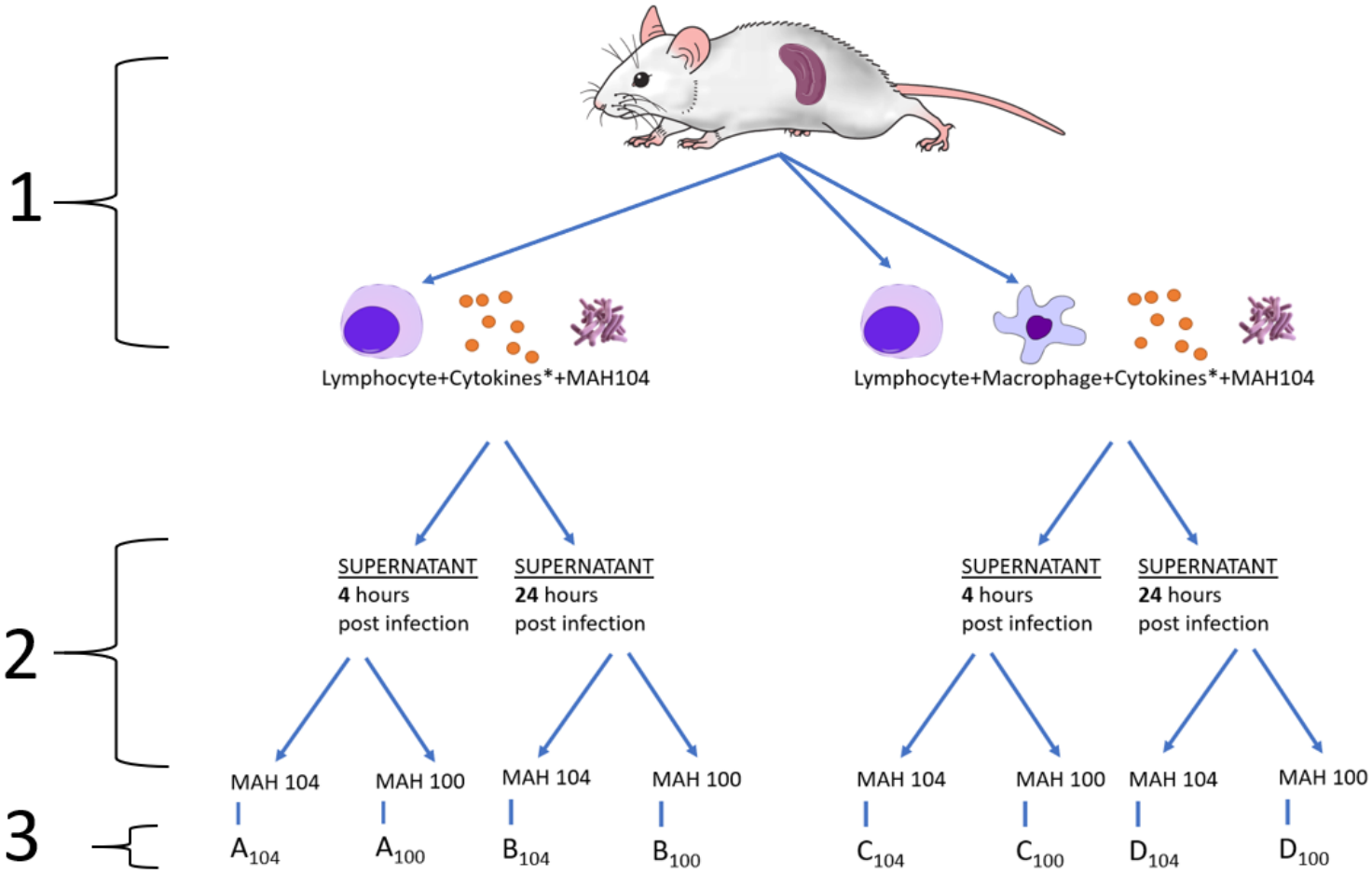

Diagram 2: Experimental Design

1) Primary lymphocytes or macrophages harvested from a mouse spleen, infected with MAH 104 and activated with *IL-12, IL-23, or IL-33
2) Supernatant from infected mouse cells added to MAH 100 and 104 RAW cell infections
3) Coded result condition groups for reference in results and discussion

Response of macrophages infected with MAH 100 to stimulation with ILC subpopulation activated supernatant

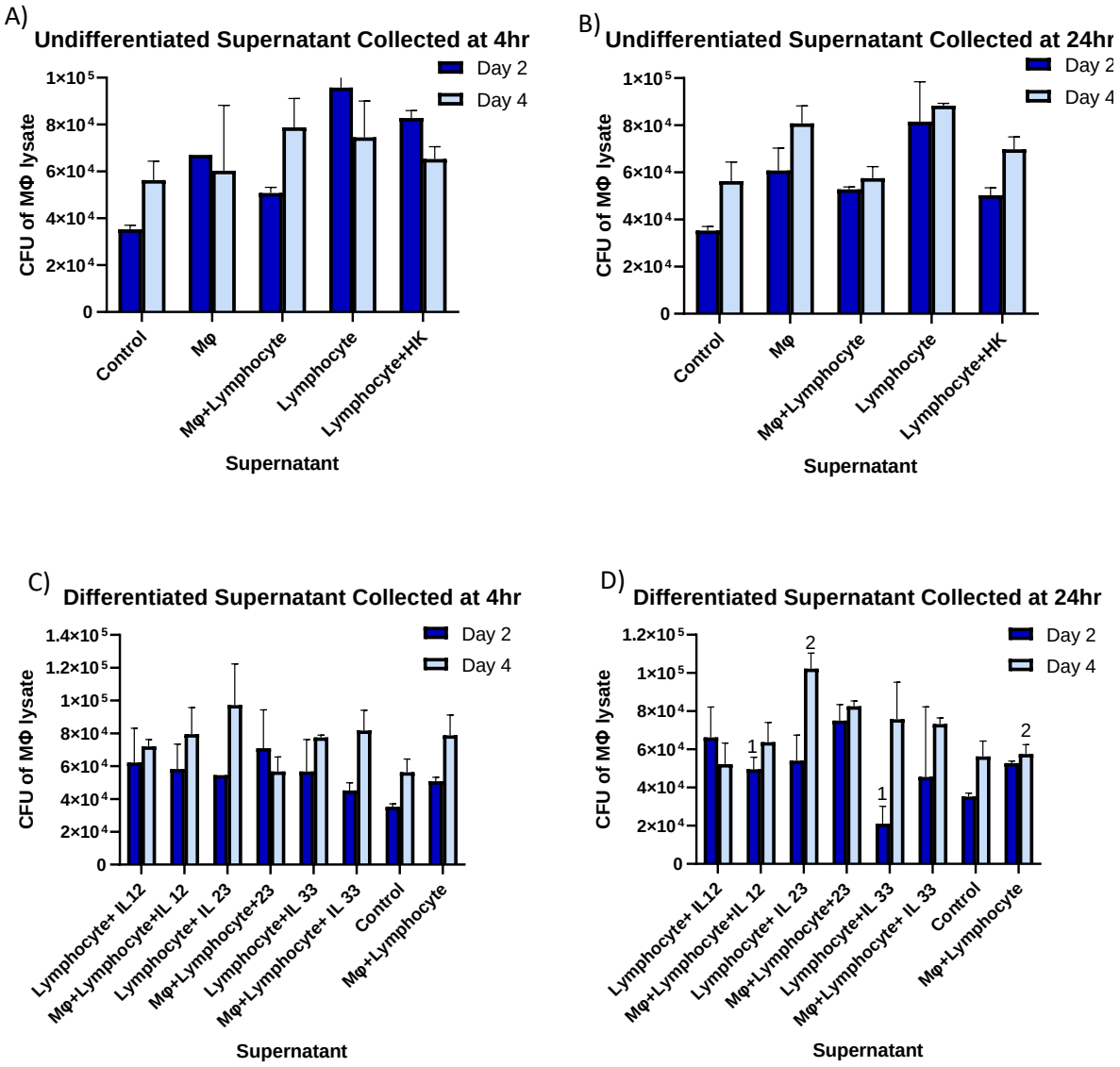

Figure 1: To determine macrophage infected with MAH 100 response to stimulation with supernatant of splenic lymphocytes exposed to IL-12, IL-23, or IL-33, a survival assay was performed. RAW cells were infected with MAH 100 and differentiated ILC subpopulation supernatants. Control wells contained fresh RPMI+10%FBS media and no supernatant. Supernatant controls included undifferentiated cells and cells infected with heat killed (HK) bacteria. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 1 independent experiment done in duplicate as well as standard deviation for the duplicates are shown here as a representative for all independent experiments (see appendix 3 for complete data of both independent experiments). Statistically significant results are indicated with corresponding numbers ($p<0.05$).

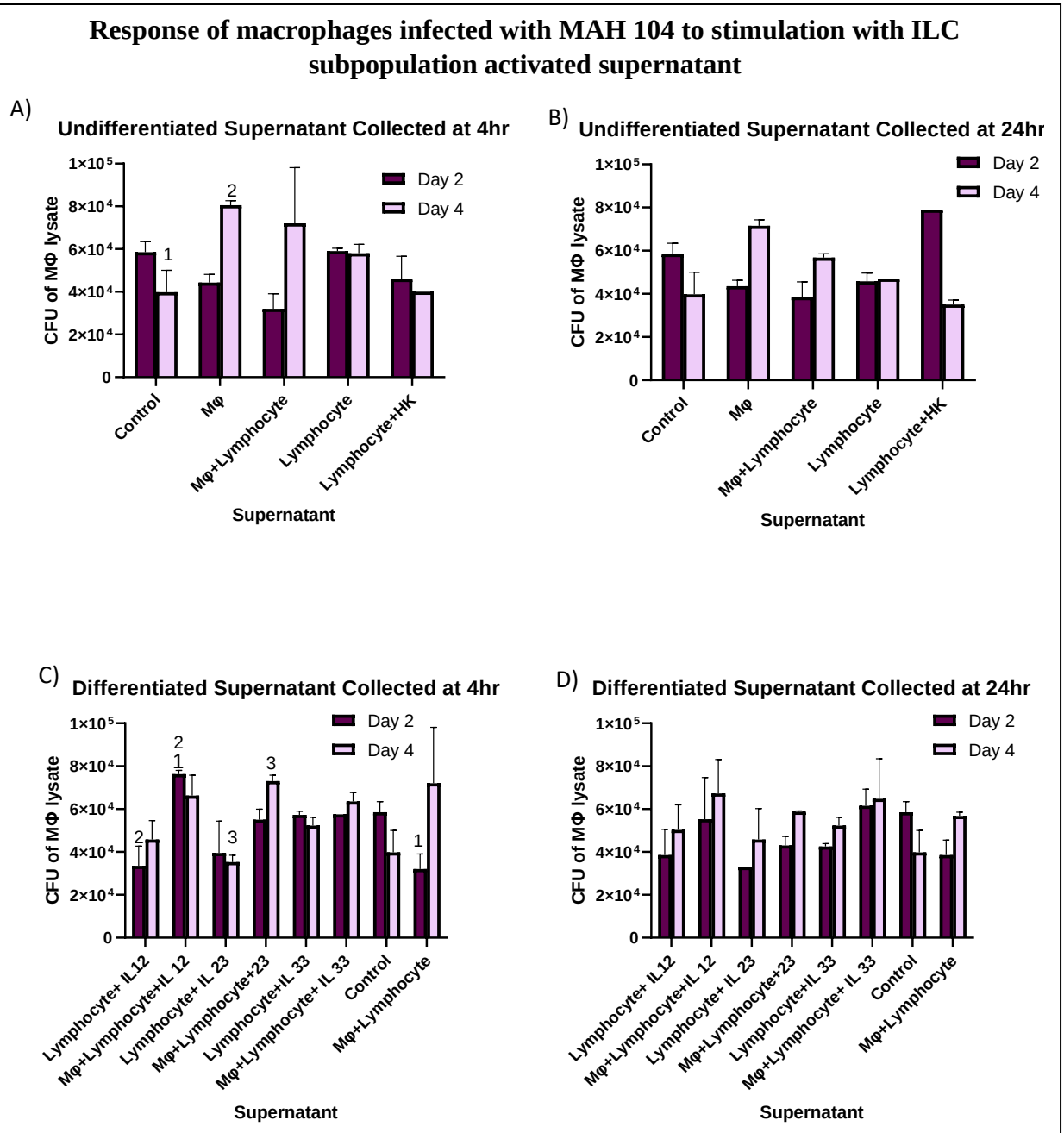

Figure 2: To determine macrophage infected with MAH 104 response to stimulation with supernatant of splenic lymphocytes treated with IL-12, IL-23, or IL-33, a survival assay was performed. RAW cells were infected with MAH 104 and differentiated ILC subpopulation supernatants. Control wells contained fresh RPMI+10% FBS media and no supernatant. Supernatant controls included undifferentiated cells and cells infected with heat killed (HK) bacteria. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 1 independent experiment done in duplicate as well as standard deviation for the duplicates are shown here as a representative for all independent experiments (see appendix 2 for complete data of both independent experiments).. Statistically significant results are indicated with corresponding numbers ($p<0.05$).

Macrophage infection in presence of recombinant cytokines

RAW cells were infected with either MAH 104 or 100, then stimulated with recombinant IL-4, IL-17, and IFNγ to determine if purified cytokines were associated with indirect activation of ILC subpopulations.

Beginning 24 hours after infection, visually IFNγ-treated wells showed irritation and change in morphology as they formed long spindles and vacuolized. This was neither seen in other wells of the recombinant cytokine experiment nor the original previous experiment containing IFNγ ILC producing subpopulations. IFNγ-treated macrophages infected with MAH 104 showed a statistically significant decrease in CFUs compared to control wells. On Day 2, IFNγ treated wells had a 52% decrease from Day 2 control cells while at Day 4 IFNγ-treated wells had a 42% decrease in bacterial load (Figure 3, panel A). This activation of macrophages was even more pronounced in MAH 100 infection where IFNγ treated wells had statistically lower CFU counts compared to control and all other recombinant cytokine treated wells (Figure 3, panel B).

The results of this assay do not align those of the initial assay using supernatant treatments since a clear difference in ability to reduce CFUs is seen between different treatments. One possible reason for this discrepancy is that the supernatant contains product(s) that are neutralizing the ILC produced cytokines. In order to investigate if this was potentially occurring a new experiment was designed that combined supernatant with cytokines.

Response of macrophages infected with MAH 104 or 100 to stimulation with recombinant cytokines

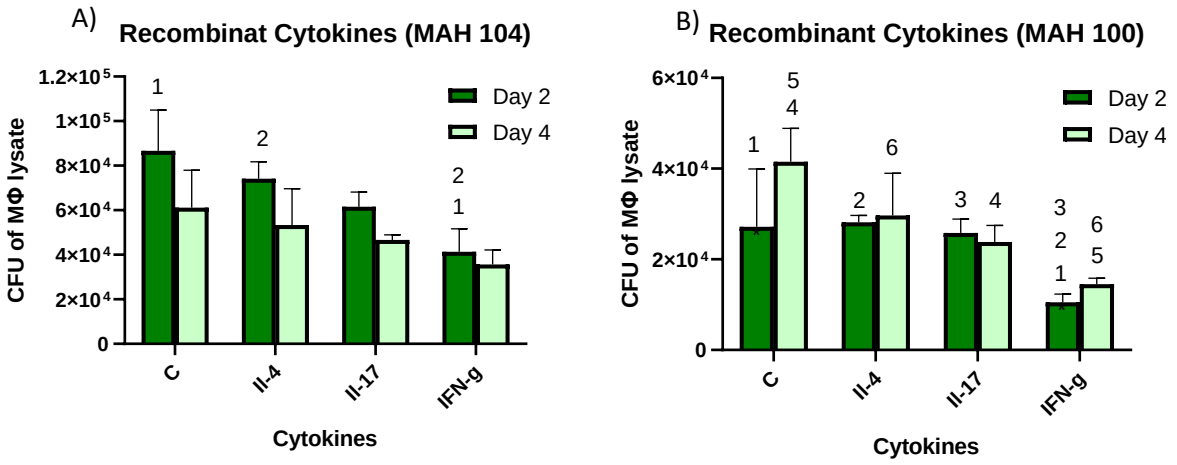

Figure 3: To determine macrophage infected with MAH 100 response to stimulation with cytokines IL-4, IL17, or IFNγ, a survival assay was performed. RAW cells were infected with MAH 100 or 104 and cytokines were added to wells. Control wells contained fresh RPMI+10% FBS media and no cytokines. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 1 independent experiment done in triplicate as well as standard deviation for the duplicates are shown here as a representative for all independent experiments (see appendix 4 and 5 for complete data of both independent experiments). Statistically significant results are indicated with corresponding numbers ($p<0.05$).

Macrophage infection in presence of recombinant cytokines and supernatant

Following infection of macrophages with MAH 104, ILC1 differentiated supernatant collected at 4 and 24 hours post infection and IFNγ were added to wells. Resulting CFUs showed statistically significant decrease of survival in all treatment groups containing supernatant derived from primary macrophages that was collected 4 hours after infection compared to controls (Figure 4, panel A). In contrast treatment with supernatant collected 24 hours post infection showed little significant ability of macrophages to kill the bacterium on either day 2 or

4 of the assay compared to controls (Figure 4, panel B). The only statistically significant supernatant condition collected at 24 hours was Day 2 lymphocyte supernatant which was 44% less than the day 2 control. In the presence of IFNγ, exposure to supernatant collected 24 hours after infection has less effect on macrophage ability to decrease bacteria in infection than supernatant collected after 4 hours (Figure 4, panel C).

Response of macrophages infected with MAH 104 to stimulation with ILC1 differentiated supernatant and IFNγ

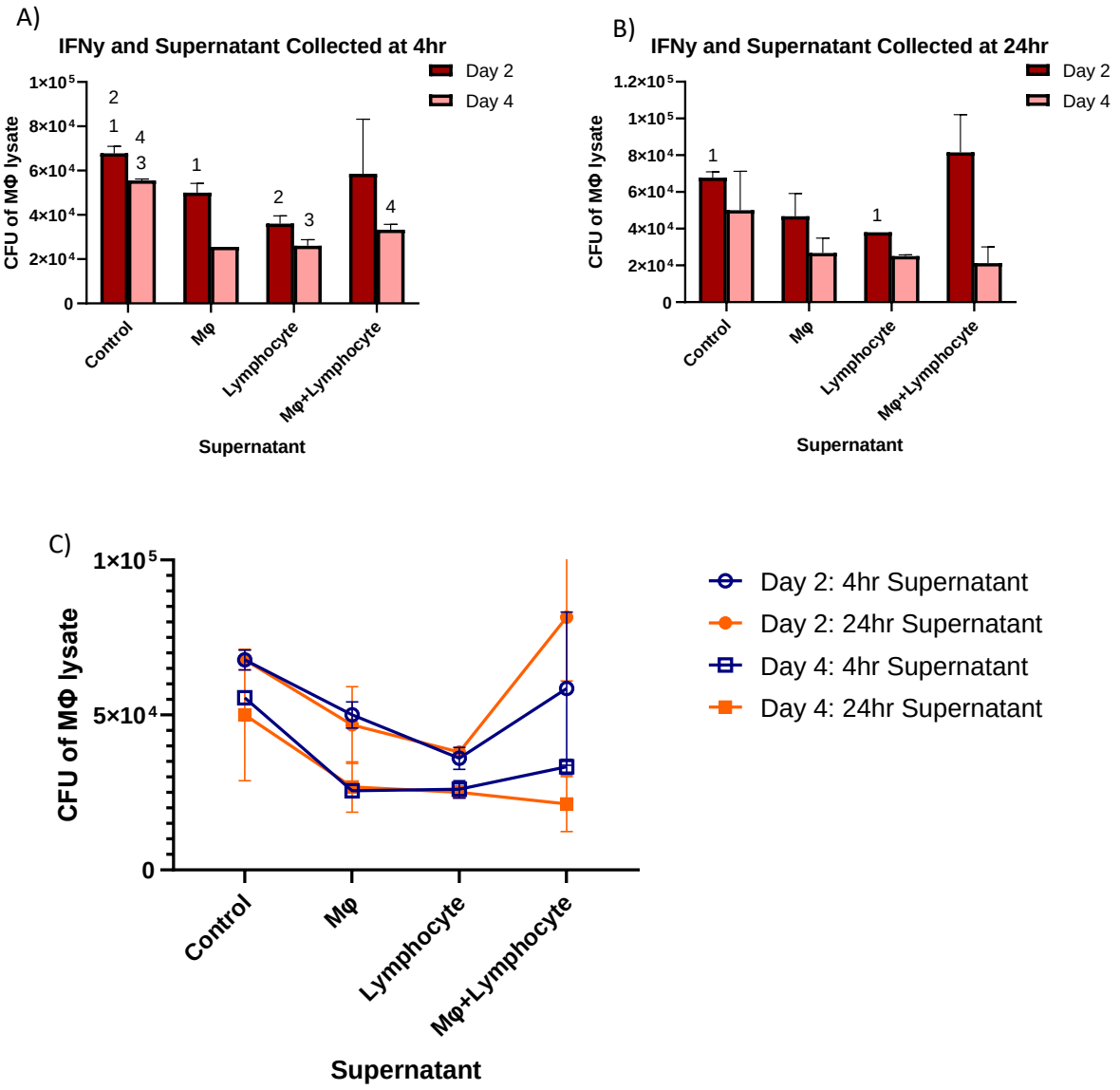

Figure 4: To determine macrophage infected with MAH 104 response to stimulation with supernatant of splenic lymphocytes treated with IL-12, as well as direct IFNγ stimulation, a survival assay was performed. RAW cells were infected with MAH 104 and differentiated ILC1 subpopulation supernatants. Control wells contained fresh RPMI+10% FBS media and no supernatant. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 1 independent experiment done in duplicate as well as standard deviation for the duplicates are shown here as a representative for all independent experiments (see appendix 6 for complete data of both independent experiments). Statistically significant results are indicated with corresponding numbers ($p<0.05$).

DISCUSSION

MAH is an important ubiquitous environmental pathogen that causes disease in immunocompromised patients[1]. After ingestion or inhalation MAH bacteria pass through mucosal layers to infect host macrophages where they survive and replicate in intracellular vacuoles[1]. While it is understood that MAH bacteria are capable of altering macrophage phagosomes to survive intracellularly, their methods of immune evasion are still largely unknown[19]. The aim of this study was to examine the role of Innate Lymphocyte Cells in MAH infections. ILCs are unspecialized cells that exist in both the mucosa and circulation where they respond to cytokine signals to differentiate and secrete cytokines to influence surrounding cells such as macrophages[7]. Our findings suggest that in the presence of macrophages and lymphocytes, MAH 104 bacterium triggers macrophages to be less responsive to ILC1 produced second order cytokine IFNγ.

The first survival assay, which used supernatant treatment on MAH 100 and 104 infection, in general showed no difference between ILC subpopulations and controls on macrophage killing abilities. MAH 100 and 104 behave differently in mice with MAH 104 being the more virulent of the two. It was hypothesized that a specific ILC subpopulation may be responsible for this difference, but results show no impact from an ILC subpopulation on either 100 or 104 infection. This result suggests that the response to *M. avium* may be different than Mycobacterium *tuberculosis*. Previous work with *M. tuberculosis* showed that ILC-3 subpopulations are able to mediate early protection against the bacterium[6]. However, while MAH and tuberculosis have many similarities, they trigger different responses in macrophages upon activation with cytokines[14]. Previous work has shown that pre-infection treatment of macrophages with IFNγ does not confer any anti-MAH response, while the same treatment

confers significant anti-*M. tuberculosis* properties to macrophages which may explain this difference[14]. Additionally, it was found in this initial assay that in general MAH 104 infected lymphocyte supernatants had statistically lower CFUs than lymphocyte and macrophage supernatants. Many of the functions of lymphocytes are to assist macrophages fight against infection, and these functions only occur when the cells are in close proximity to macrophages. In contrast, MAH 100 infections showed no significant difference at 4hr and limited significance at 24 hours. This data alternatively suggests that MAH 104 interferes with normal macrophage and lymphocyte interactions when both are present.

In order to further explore the potential suppressive effects of the supernatant, a second survival assay looking at direct macrophage activation of MAH 100 and 104 infected macrophages with recombinant cytokines was performed. This was carried out using the second order cytokines expected to be produced and released in the supernatant for each ILC subpopulation in order to establish the anticipated effect of the supernatant on macrophage infection. Results showed a significant decrease in CFUs for IFNγ treated MAH 104 and an even greater decrease in CFUs for MAH 100 infections. IFNγ is known to upregulate anti-MAH 104 activity in macrophages and not be produced in significant quantities in MAH 100 infections[3,19]. However even though MAH 100 infections don't typically produce IFNγ it is not unexpected that is had a strong impact on macrophage ability to reduce infection because IFNγ supports macrophage ability to clear intracellular pathogens.

In order to look at the impact of IFNγ stimulation directly in conjunction with ILC-1 (IFNγ-producing) supernatant on MAH 104 infections, a survival assay was preformed where both were added post infection. Wells with supernatant collected 4 hours post infection show a significant decrease in macrophage killing ability, whereas wells with supernatant collected 24

hours post infection show significantly less ability for macrophages to limit MAH 104 infection. These results suggest that when exposed to MAH 104 the effect of IFNγ is suppressed by the actions of the supernatant. The presence of this effect is most apparent in supernatant collected 24 hours post infection suggesting that this is a response that occurs over time as host cells and bacteria interact.

As demonstrated in the assay of macrophage infection in presence of recombinant cytokines, as well as literature, IFNγ clearly plays a role in antimycobacterial activity[15,19]. However, this effect was neither apparent in the original assay of macrophage infection in presence of supernatant nor the final assay of supernatant in conjunction with direct IFNγ stimulation. Literature analysis shows that IFNγ treatment of MAH macrophages confers less macrophage bacteriostatic abilities than macrophages infected with *M. tuberculosis*[6]. In the scope of this study, this discrepancy of IFNγ impact on MAH infection may be explained by the presence of some product(s) produced upon activation of ILC subpopulation in the presence of macrophages and lymphocytes that block the effect of IFNγ.

There are many possibilities regarding what is responsible for the decreased impact of supernatant on IFNγ. It has been shown in literature that *Mycobacterium avium* infections cause an increased output of IL-10 that is implicated in MAH pathogenesis[16,17]. When treated with IL-10 neutralizing antibodies mouse resistance to infections is conferred[16,17]. Additionally similar results are seen with mice treated with TGF-beta and TGF-beta neutralizing infusions. where TGF-beta leads to increased MAH growth that is counteracted when neutralized[18]. While IL-10 synthesis and secretion begins shortly after initiation of infection, TGF-beta levels are first detected at day 3[19]. This is one potential difference between the difference in supernatant

collected 2 days post infections versus 4 days that leads to blockage of IFNγ effect and decreased anti-MAH capabilities.

Further work needs to be done to address the limitations in the scope of this study. Firstly, as introduced earlier, splenic and lung derived lymphocytes interact with different cells to activate and differentiate. While these differences in activation are known, it has not been determined whether once activated they have the same outputs. Spleen derived lymphocytes were used in this research because of their relative ease to harvest compared to lung derived lymphocytes, but future work may benefit from directly comparing the results of this work when done with spleen versus lung derived lymphocytes. Secondly, future work should measure the levels of different ILC produced cytokines in supernatant as well as resulting supernatant from macrophage infection in presence of recombinant cytokines and supernatant to determine whether cytokines such as IFNγ are present in effective levels or not present at all.

MAH infections do not cause illness in the average individual, but for AIDS patients and individuals with chronic lung disease MAH infections can become serious and expensive, requiring long treatments with multiple drugs. The role of ILCs in these infections are still not fully known. This study has shown that MAH infection can interfere with ILC-1 activity and ultimately diminish the effects of IFNγ. In the future better understanding of ILC roles in MAH infections and immune responses will lead to better treatment options.

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APPENDIX

Supernatant Key

Supernatant Key	Supernatant Condition	ILC Subpopulation
M 4hr	Primary mouse macrophage collected 4 hours post infection	undifferentiated
M 24hr	Primary mouse macrophage collected 24 hours post infection	undifferentiated
M+L 4hr	Primary mouse macrophage and lymphoytes collected 4 hours post infection	undifferentiated
M+L 24hr	Primary mouse macrophage and lymphoytes collected 24 hours post infection	undifferentiated
M+L+IL12 4hr	Primary mouse macrophage and lymphoytes stimulated with IL-12 collected 4 hours post infection	ILC1
M+L+IL12 24hr	Primary mouse macrophage and lymphoytes stimulated with IL-12 collected 24 hours post infection	ILC1
M+L+IL23 4hr	Primary mouse macrophage and lymphoytes stimulated with IL-23 collected 4 hours post infection	ILC3
M+L+IL23 24hr	Primary mouse macrophage and lymphoytes stimulated with IL-23 collected 24 hours post infection	ILC3
M+L+IL33 4hr	Primary mouse macrophage and lymphoytes stimulated with IL-33 collected 4 hours post infection	ILC2
M+L+IL33 24hr	Primary mouse macrophage and lymphoytes stimulated with IL-33 collected 24 hours post infection	ILC2
L 4hr	Primary mouse lymphocytes collected 4 hours post infection	undifferentiated
L 24hr	Primary mouse lymphocytes collected 24 hours post infection	undifferentiated
L+IL12 4 hr	Primary mouse lymphocytes stimulated with IL-12 collected 4 hours post infection	ILC1
L+IL12 24hr	Primary mouse lymphocytes stimulated with IL-12 collected 24 hours post infection	ILC1
L+IL23 4hr	Primary mouse lymphocytes stimulated with IL-23 collected 4 hours post infection	ILC2
L+IL23 24hr	Primary mouse lymphocytes stimulated with IL-23 collected 24 hours post infection	ILC2
L+IL33 4hr	Primary mouse lymphocytes stimulated with IL-33 collected 4 hours post infection	ILC3
L+IL33 24hr	Primary mouse lymphocytes stimulated with IL-33 collected 24 hours post infection	ILC3
L+HK 4hr	Primary mouse lymphocytes infected with heat killed MAH 104 collected 44 hours post infection	undifferentiated
L+HK 24hr	Primary mouse lymphocytes infected with heat killed MAH 104 collected 24 hours post infection	undifferentiated

Appendix 1: To create supernatant, wells containing either primary mouse lymphocytes, macrophages, or lymphocytes and macrophages were infected with MAH 104 and either differentiated with IL-12, 23, and 33 into ILC subpopulations or left undifferentiated. Here a key to the supernatant conditions and descriptions is provided.

CFUs from Macrophage Infection with MAH 104 and Supernatant Treatment

	104 Day 2				104 Day 4			
Supernatant	CFU #1	CFU #2	average	sd	CFU #1	CFU #2	average	sd
M 4hr	5.08E+04	5.05E+04	**5.06E+04**	**1.77E+02**	3.63E+04	8.05E+04	**5.84E+04**	**3.13E+04**
M 24hr	6.08E+04	4.35E+04	**5.21E+04**	**1.22E+04**	5.33E+04	7.15E+04	**6.24E+04**	**1.29E+04**
M+L 4hr	5.23E+04	3.20E+04	**4.21E+04**	**1.43E+04**	4.70E+04	7.20E+04	**5.95E+04**	**1.77E+04**
M+L 24hr	4.45E+04	3.85E+04	**4.15E+04**	**4.24E+03**	NA	5.68E+04	**5.68E+04**	**NA**
M+L+IL12 4hr	7.98E+04	7.63E+04	**7.80E+04**	**2.47E+03**	5.50E+04	6.63E+04	**6.06E+04**	**7.95E+03**
M+L+IL12 24hr	6.33E+04	5.53E+04	**5.93E+04**	**5.66E+03**	5.55E+04	6.73E+04	**6.14E+04**	**8.31E+03**
M+L+IL23 4hr	4.83E+04	5.50E+04	**5.16E+04**	**4.77E+03**	5.30E+04	7.30E+04	**6.30E+04**	**1.41E+04**
M+L+IL23 24hr	5.03E+04	4.30E+04	**4.66E+04**	**5.13E+03**	4.95E+04	5.88E+04	**5.41E+04**	**6.54E+03**
M+L+IL33 4hr	5.45E+04	5.75E+04	**5.60E+04**	**2.12E+03**	5.18E+04	6.35E+04	**5.76E+04**	**8.31E+03**
M+L+IL33 24hr	4.48E+04	6.15E+04	**5.31E+04**	**1.18E+04**	6.75E+04	6.48E+04	**6.61E+04**	**1.94E+03**
L 4hr	7.05E+04	5.90E+04	**6.48E+04**	**8.13E+03**	5.35E+04	5.80E+04	**5.58E+04**	**3.18E+03**
L 24hr	7.20E+04	4.58E+04	**5.89E+04**	**1.86E+04**	4.95E+04	4.70E+04	**4.83E+04**	**1.77E+03**
L+IL12 4 hr	4.95E+04	3.35E+04	**4.15E+04**	**1.13E+04**	5.05E+04	4.58E+04	**4.81E+04**	**3.36E+03**
L+IL12 24hr	3.88E+04	3.85E+04	**3.86E+04**	**1.77E+02**	3.85E+04	5.03E+04	**4.44E+04**	**8.31E+03**
L+IL23 4hr	7.13E+04	3.95E+04	**5.54E+04**	**2.25E+04**	5.23E+04	3.53E+04	**4.38E+04**	**1.20E+04**
L+IL23 24hr	4.95E+04	3.30E+04	**4.13E+04**	**1.17E+04**	5.30E+04	4.58E+04	**4.94E+04**	**5.13E+03**
L+IL33 4hr	2.68E+04	5.73E+04	**4.20E+04**	**2.16E+04**	6.75E+04	5.60E+04	**6.18E+04**	**8.13E+03**
L+IL33 24hr	3.20E+04	4.25E+04	**3.73E+04**	**7.42E+03**	5.23E+04	5.23E+04	**5.23E+04**	**0.00E+00**
L+HK 4hr	4.70E+04	4.60E+04	**4.65E+04**	**7.07E+02**	5.90E+04	4.00E+04	**4.95E+04**	**1.34E+04**
L+HK 24hr	3.78E+04	7.90E+04	**5.84E+04**	**2.92E+04**	5.95E+04	3.50E+04	**4.73E+04**	**1.73E+04**
control	8.68E+04	5.85E+04	**7.26E+04**	**2.00E+04**	7.45E+04	3.98E+04	**5.71E+04**	**2.46E+04**

Appendix 2: To determine macrophage infected with MAH 104 response to stimulation with supernatant of splenic lymphocytes treated with IL-12, IL-23, or IL-33 a survival assay was performed.. RAW cells were infected with MAH 104 and differentiated ILC subpopulation supernatants. Control wells contained fresh RPMI media and no supernatant. Supernatant controls included undifferentiated cells and cells infected with heat killed (HK) bacteria. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 2 independent experiments done in triplicate is shown as well as standard deviation for the 2 independent experiments.

CFUs from Macrophage Infection with MAH 100 and Supernatant Treatment

	100 Day 2				100 Day 4			
Supernatant	CFU #1	CFU #2	average	sd	CFU #1	CFU #2	average	sd
M 4hr	7.18E+04	6.70E+04	**6.94E+04**	**3.36E+03**	7.85E+04	6.03E+04	**6.94E+04**	**1.29E+04**
M 24hr	6.23E+04	6.08E+04	**6.15E+04**	**1.06E+03**	8.40E+04	8.08E+04	**8.24E+04**	**2.30E+03**
M+L 4hr	6.30E+04	5.08E+04	**5.69E+04**	**8.66E+03**	8.08E+04	7.88E+04	**7.98E+04**	**1.41E+03**
M+L 24hr	6.68E+04	5.28E+04	**5.98E+04**	**9.90E+03**	5.70E+04	5.75E+04	**5.73E+04**	**3.54E+02**
M+L+IL12 4hr	7.88E+04	5.83E+04	**6.85E+04**	**1.45E+04**	8.40E+04	7.95E+04	**8.18E+04**	**3.18E+03**
M+L+IL12 24hr	6.78E+04	4.95E+04	**5.86E+04**	**1.29E+04**	8.60E+04	6.38E+04	**7.49E+04**	**1.57E+04**
M+L+IL23 4hr	4.85E+04	7.10E+04	**5.98E+04**	**1.59E+04**	5.08E+04	5.68E+04	**5.38E+04**	**4.24E+03**
M+L+IL23 24hr	5.20E+04	7.50E+04	**6.35E+04**	**1.63E+04**	5.23E+04	8.25E+04	**6.74E+04**	**2.14E+04**
M+L+IL33 4hr	5.80E+04	4.53E+04	**5.16E+04**	**9.02E+03**	9.95E+04	8.18E+04	**9.06E+04**	**1.26E+04**
M+L+IL33 24hr	6.83E+04	4.55E+04	**5.69E+04**	**1.61E+04**	8.75E+04	7.33E+04	**8.04E+04**	**1.01E+04**
L 4hr	6.33E+04	9.58E+04	**7.95E+04**	**2.30E+04**	7.33E+04	7.45E+04	**7.39E+04**	**8.84E+02**
L 24hr	6.58E+04	8.15E+04	**7.36E+04**	**1.11E+04**	6.53E+04	8.83E+04	**7.68E+04**	**1.63E+04**
L+IL12 4 hr	4.33E+04	6.23E+04	**5.28E+04**	**1.34E+04**	5.98E+04	7.20E+04	**6.59E+04**	**8.66E+03**
L+IL12 24hr	4.48E+04	6.63E+04	**5.55E+04**	**1.52E+04**	5.00E+04	5.23E+04	**5.11E+04**	**1.59E+03**
L+IL23 4hr	6.53E+04	5.45E+04	**5.99E+04**	**7.60E+03**	6.90E+04	9.70E+04	**8.30E+04**	**1.98E+04**
L+IL23 24hr	5.35E+04	5.40E+04	**5.38E+04**	**3.54E+02**	6.65E+04	1.02E+05	**8.43E+04**	**2.51E+04**
L+IL33 4hr	5.70E+04	5.68E+04	**5.69E+04**	**1.77E+02**	5.58E+04	7.75E+04	**6.66E+04**	**1.54E+04**
L+IL33 24hr	5.68E+04	2.10E+04	**3.89E+04**	**2.53E+04**	5.78E+04	7.58E+04	**6.68E+04**	**1.27E+04**
L+HK 4hr	5.00E+04	8.28E+04	**6.64E+04**	**2.32E+04**	6.43E+04	6.53E+04	**6.48E+04**	**7.07E+02**
L+HK 24hr	6.60E+04	5.03E+04	**5.81E+04**	**1.11E+04**	5.85E+04	6.98E+04	**6.41E+04**	**7.95E+03**
control	6.38E+04	3.53E+04	**4.95E+04**	**2.02E+04**	9.63E+04	5.63E+04	**7.63E+04**	**2.83E+04**

Appendix 3: To determine macrophage infected with MAH 100 response to stimulation with supernatant of splenic lymphocytes treated with IL-12, IL-23, or IL-33 a survival assay was performed. RAW cells were infected with MAH 104 and differentiated ILC subpopulation supernatants. Control wells contained fresh RPMI media and no supernatant. Supernatant controls included undifferentiated cells and cells infected with heat killed (HK) bacteria. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 2 independent experiments done in triplicate is shown as well as standard deviation for the 2 independent experiments.

CFUs from Macrophage Infection with MAH 104 and Recombinant Cytokine Treatment

	104 Day 2				104 Day 4			
Supernantant	CFU #1	CFU #2	average	SD	CFU #1	CFU #2	average	SD
C	8.67E+04	5.72E+04	**7.19E+04**	**2.09E+04**	6.12E+04	5.55E+04	**5.83E+04**	**4.01E+03**
Il-4	7.42E+04	4.63E+04	**6.03E+04**	**1.97E+04**	5.33E+04	3.77E+04	**4.55E+04**	**1.11E+04**
Il-17	6.15E+04	3.93E+04	**5.04E+04**	**1.57E+04**	4.67E+04	3.25E+04	**3.96E+04**	**1.00E+04**
IFN-g	4.13E+04	4.62E+04	**4.38E+04**	**3.42E+03**	3.57E+04	1.37E+04	**2.47E+04**	**1.56E+04**

Appendix 4: To Determine the effect of direct cytokine activation of macrophage control of MAH 104 infection, a survival assay was performed. RAW cells were infected with MAH 104 and IL-4, IL-17, or IFNy cytokine treatments were added to each well. Control wells contained fresh RPMI media and no cytokine addition. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 2 independent experiments done in triplicate is shown as well as standard deviation for the 2 independent experiments.

CFUs from Macrophage Infection with MAH 100 and Recombinant Cytokine Treatment

	100 Day 2				100 Day 4			
Supernantant	CFU #1	CFU #2	average	SD	CFU #1	CFU #2	average	SD
C	9.93E+04	2.72E+04	**6.33E+04**	**5.10E+04**	2.85E+04	4.15E+04	**3.50E+04**	**9.19E+03**
Il-4	4.83E+04	2.82E+04	**3.83E+04**	**1.43E+04**	3.10E+04	2.97E+04	**3.03E+04**	**9.43E+02**
Il-17	2.42E+04	2.58E+04	**2.50E+04**	**1.18E+03**	2.30E+04	2.38E+04	**2.34E+04**	**5.89E+02**
IFN-g	1.98E+04	1.05E+04	**1.52E+04**	**6.60E+03**	1.45E+04	1.45E+04	**1.45E+04**	**0.00E+00**

Appendix 5: To Determine the effect of direct cytokine activation of macrophage control of MAH 104 infection, a survival assay was performed. RAW cells were infected with MAH 100 and IL-4, IL-17, or IFNy cytokine treatments were added to each well. Control wells contained fresh RPMI media and no cytokine addition. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 2 independent experiments done in triplicate is shown as well as standard deviation for the 2 independent experiments.

CFUs from Macrophage Infection with MAH 100 and Recombinant Cytokine Treatment

	Day 2				Day 4			
Supernatant	CFU #1	CFU #2	average	sd	CFU #1	CFU #2	average	sd
Control	6.83E+04	6.78E+04	**6.80E+04**	**3.54E+02**	2.48E+04	4.55E+04	**3.51E+04**	**1.47E+04**
Macrophage T-4	5.30E+04	5.00E+04	**5.15E+04**	**2.12E+03**	2.38E+04	2.55E+04	**2.46E+04**	**1.24E+03**
Macrophage T-24	5.30E+04	4.68E+04	**4.99E+04**	**4.42E+03**	3.03E+04	2.68E+04	**2.85E+04**	**2.47E+03**
T-cell+IL 12 4hr	4.35E+04	3.60E+04	**3.98E+04**	**5.30E+03**	3.25E+04	2.60E+04	**2.93E+04**	**4.60E+03**
T-cell+IL 12 24hr	3.25E+04	3.80E+04	**3.53E+04**	**3.89E+03**	2.88E+04	2.50E+04	**2.69E+04**	**2.65E+03**
T-cell+IL 12 4hr	4.55E+04	5.85E+04	**5.20E+04**	**9.19E+03**	3.75E+04	3.33E+04	**3.54E+04**	**3.01E+03**
T-cell+IL 12 24hr	6.60E+04	8.15E+04	**7.38E+04**	**1.10E+04**	3.35E+04	2.13E+04	**2.74E+04**	**8.66E+03**
Heat Killed Bacteria 4hr	3.05E+04	2.83E+04	**2.94E+04**	**1.59E+03**	2.63E+04	9.50E+03	**1.79E+04**	**1.18E+04**

Appendix 6: To Determine the effect of ILC1 (IFNy producing) activated lymphocytes in conjunction with direct IFNy stimulation on macrophage control of MAH 104 infection, a survival assay was performed. RAW cells were infected with MAH 104 and IL-12 stimulated supernatant (ILC1) were added to wells in addition to IFNy. Control wells contained fresh RPMI media and no cytokine addition as wells with control supernatants that were no differentiated into ILC1. Wells were lysed at 2 days and 4 days to determine intracellular bacteria at each time point as CFUs. CFU counts from 2 independent experiments done in triplicate is shown as well as standard deviation for the 2 independent experiments.