Impact of obesity on immune responses to influenza virus infection

By

AARAM A KUMAR

Thesis
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in

Microbiology & Immunology
May 2016
Nashville, Tennessee

Approved:

Luc Van Kaer, PhD

Ray Stokes Peebles, MD

To my mother, for always believing in me.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my project advisor Dr. Luc Van Kaer for providing me an opportunity to work under his inspiring guidance. I am extremely thankful to him for his mentorship, valuable suggestions and encouragement throughout the course of the project.

I would also like to thank members of my thesis advisory committee: Dr. R. Stokes Peebles, Dr. Edward R. Sherwood, Dr. David Aronoff, and Dr. Alyssa Hasty for their time, critical comments and suggestions.

I would like to thank the Vanderbilt International Scholar Program for financial support.

I would also like to express my heartfelt gratitude to Dr. Lan Wu and Dr. Vrajesh Parekh for their valuable input to my project during several discussions.

I would like to thank my friends and family for their love, affection and support.

.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

CHAPTER 1

Introduction

Obesity and Influenza

Obesity has become a major health concern in the United States with over 60% of the Americans currently being overweight or obese. It increases the risk for a number of disorders such as cardiovascular diseases, metabolic syndrome and Type II diabetes. It also exacerbates respiratory disorders such as chronic obstructive pulmonary disease and asthma. While it is known that obesity causes chronic low-grade inflammation and alters immune responses, how it affects immune function during infections is not clearly understood. Apart from the physical issues that may arise due to obesity – lowered lung capacity, decreased mobility etc. – obesity directly influences immune responses in the lungs. Since a robust pulmonary immune response is required for successful clearance of influenza virus upon exposure, such an effect can be dangerous to the individual and increase risk of infection. Previous studies have explored the link between obesity and *Clostridium difficile* and H1N1, H5N1 and H3N2 influenza virus infections[1,2,3,4]. These studies demonstrated a role of obesity as an independent risk factor in these infections. Influenza causes approximately 36,000 deaths in the United States every year and obese individuals appear to be at greater risk of infection by the virus[5,6]. Studies have demonstrated increased mortality, poor clinical outcomes and longer recovery times for obese patients. Murine studies have shown that obesity profoundly impairs vaccine protection against H1N1[7]. Obesity also affects heterologous immunity against different influenza virus strains[8]. Dendritic cell impairment, poor antigen presentation, impaired

$CD8^+$ T cell function and reduced NK cell cytotoxicity all have been implicated in these worsened outcomes[9,10,11,12,13]. Molecules such as leptin and adiponectin that are altered by obesity have been suggested to induce some of the differential outcomes[14,15]. Recent studies have found that obesity affects the metabolic profile in the lungs and it has been suggested that this potentially contributes to impaired immune responses[16]. However, several of these studies were correlative in nature, or had small sample sizes and did not closely investigate the role of macrophages. Among the different cell types that modulate pulmonary immunity, alveolar macrophages (AMs) are particularly important because they not only initiate immune responses but also prevent excessive inflammation[17]. Cytokines secreted by AMs during initial stages of infection, especially type I interferons such as interferon-α (IFN-α) and IFN-β and IL-6 are essential for clearance of viruses that infect the respiratory tract. Type I interferons can influence dendritic cells and thus indirectly impact T cell activation. The secretion of these cytokines by AMs is thus not only important for initial innate immune responses, but also crucial for proper adaptive immunity. Hence it is important to understand how AM functions are altered during obesity. So I decided to examine how obesity affects immune responses to influenza A virus infection using diet-induced obese mice while focusing on the role of alveolar macrophages.

Rationale

Alveolar macrophages (AMs) are the first line of defense against respiratory pathogens. They phagocytose invading pathogens, recruit other cells of the immune system such as neutrophils and NK cells to the lung and secrete cytokines that help to eliminate the

invader[18]. They also keep other cell types of the immune system in check to avoid them from generating an overwhelming immune response, which can be detrimental to the host[19]. During the last decade, specifically after the H1N1 strain influenza pandemic of 2009, evidence has emerged to demonstrate that obesity increases the risk for infection by influenza A virus[20,21,22,23]. Obese individuals were hospitalized at a higher rate compared to their lean counterparts[24]. They also responded poorly to vaccination and had worse outcomes with longer hospitalization time and increased mortality[25,26]. However, how obesity affects immune responses in the lungs against influenza is incompletely understood. Hence it is of paramount importance to study the underlying factors that contribute to this enhanced susceptibility to infection and disease. I propose to investigate how AMs influence immune responses to influenza virus infection during obesity. *I hypothesized that obesity impairs AM function and this contributes to suboptimal immune responses to influenza virus infection.*

Impact of obesity on alveolar macrophage responses during influenza virus infection

I hypothesized that obesity alters AM polarization and this contributes to worsened outcomes in obese individuals. To further test this hypothesis, I (i) Evaluated the inflammatory properties of AMs (pro- vs anti-inflammatory) during influenza virus infection in obese and lean mice. (ii) Depleted AMs and evaluated progression of infection and response to the infection in both groups.

Mechanism

How does obesity modulate alveolar macrophage responses to influenza infection?

Once I determined the extent to which obesity influences AM responses, I focused on elucidating the mechanism. AM development and function require activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ)[27]. It affects AM polarization, overall inflammatory responses and outcomes during bacterial infection[28,29,30,31]. The surface signaling molecule Galectin 3 (Gal-3) that plays important roles in immune regulation activates PPAR-γ and drives alternative activation of macrophages[32,33]. Hence *I hypothesized that differential PPAR-γ activation in obese AMs induced by Galectin 3 (Gal-3) could be a reason for the observed change in overall immune response.* Both Gal-3 and PPAR-γ have been shown to influence TLR signaling[34,35,36,37]. Hence a change in these key molecules along with the chronic low grade inflammation in obese individuals could lead to dysfunctional Toll-Like Receptor (TLR) signaling by AM[38]. *I hypothesized that obesity causes TLR desensitization, which impairs their ability to initiate an optimal immune response during infection*. To test these possibilities, I decided to: (i) Modulate PPAR-γ activation status chemically using ligands and assess infection outcomes. (ii) Stimulate TLRs on AM using specific ligands and compare how prior stimulation of TLRs affects alveolar macrophage responses to respiratory challenges in obese and lean mice. If the worsened outcomes in obese individuals during infection are regulated via TLR or PPAR-γ dependent mechanisms, therapeutic intervention using small molecule modulators of these key regulatory proteins will be possible.

Alveolar Macrophages

Since AMs initiate as well as regulate immune response to respiratory pathogens, it is crucial to understand to what extent their response is modulated by obesity. Further, whether a cell type is beneficial (or detrimental) is important from a therapeutic point of view. Hence I decided to address these questions by determining the extent to which obesity affects alveolar macrophage responses during influenza virus infection.

I also decided to delve into the mechanistic aspects and delineate how obesity potentially modulates AM responses to influenza virus infection. Knowledge of the mechanism will provide new insight on effects of obesity on the pulmonary immune system and immune responses to respiratory infections. This work is also of clinical relevance because, should our hypothesis be confirmed, worsened disease outcomes during obesity could be alleviated by targeted activation or suppression of AMs. If the differential outcomes in obese individuals during infection are mediated/regulated via TLR- and/or PPAR-γ-dependent mechanisms, small molecule modulators of these key regulatory proteins may be used therapeutically.

Obesity affects immune cell functions, but little is known about its effects on key metabolic processes within the cell type such as fatty acid oxidation, lipolysis, glycolysis and oxidative phosphorylation. This is relevant since it has been shown that macrophages as well as T cells use lipolysis as a mechanism to differentiate into cells with specific inflammatory phenotypes. AMs, like other subsets of macrophages, can exhibit a predominantly pro-inflammatory (M1) or anti-inflammatory (M2) phenotype[62,63,64]. While M1 macrophages use glycolysis as their predominant source of energy, oxidative phosphorylation is used by M2 macrophages to a greater extent. M1 macrophages are

associated with higher levels of IL-6 and TNF-α production and have a higher iNOS/Arginase ratio, whereas M2 macrophages are typically characterized by higher levels of CD206, CD163 and prostaglandin E2 production, and a lower iNOS/Arginase ratio[39,40,41]. Macrophages increase lipolysis to enhance fatty acid oxidation to assume an M2-like phenotype[42]. For $CD8^+$ T cells, lipolysis is essential to progress into a memory T cell phenotype[43]. Evidence from multiple studies also indicates that excessive lipids accumulated in the bloodstream due to a high-fat diet can circulate to the lung and taken up by resident cells such as AMs. Such metabolic changes can alter pulmonary immune responses in otherwise healthy, wild type (C57BL/6) mice. For instance, mice fed with a high cholesterol diet had a lesser degree of migration of peripheral mononuclear cells (PMNs) to the lung in response to lipopolysaccharide (LPS) as well as the bacterium *Klebsiella pneumoniae*, and were diminished in their ability to clear bacteria from the lung[44,45,46]. I believe that investigating the metabolic differences between AMs from obese and lean mice elaborates the reason behind the AM phenotype change (there are more M2-like AMs in mice fed a high-fat diet) and this could be a cause for increased susceptibility to influenza. It will also highlight the role changes in cellular metabolism play in immune responses to infection outcomes.

This project is one of the first to address the mechanism by which obesity alters alveolar macrophage responses. Considering the magnitude of the current obesity pandemic and the extensive way in which it influences the immune system, it is imperative to study how obesity affects immune responses to infections. Here I used diet-induced obese (DIO) mice in a C57BL/6J background (fed a 60% fat diet) and mice from the same background fed a regular low-fat diet for the experiments. This is the favored model as compared with

mice or rats genetically altered to induce obesity (such as the ob/ob mice) because metabolic parameters like glucose tolerance and insulin resistance in the former model mimic human obesity more closely[47,48]. Moreover, while many of the mutations used to genetically induce obesity in mice are not relevant to humans, diet induced obesity is more similar to the human scenario. The influenza A virus used in this study is a mouse-adapted H1N1 strain (PR8). The use of this mouse model and this virus strain is ideal as the responses and outcomes upon infection are similar to those observed in humans. I hypothesized that obesity induces changes in AM polarization and consequently impairs initial immune responses against the virus. This potentially leads to dampened adaptive immunity as well and thus contributes to worsened disease outcomes. I expect that this project will bridge the gap in our knowledge of how obesity affects pulmonary immune responses. While many of the techniques I used in this project are employed by our laboratory and other researchers to study either obesity or infections, I integrated them into a combined obesity-respiratory infection context.

CHAPTER 2

Materials and Methods

The aims span the broad theme of alveolar macrophage responses to influenza virus infection during obesity and are planned to understand: (1) the extent to which obesity influences AM responses to influenza virus infection, (2) how obesity modulates AM responses to infection.

Model system and diet

For all infection experiments, unless otherwise stated, C57BL/6 wild type male mice of 14 weeks (18 weeks for some studies) of age were used. High-fat diet feeding started when the mice were 6 weeks old, and they were kept on diet for 8 weeks before being infected with influenza virus PR/8 strain. The viral dose of infection was set at 6.5 x 10^3 PFU of Influenza A/PR8 virus diluted in PBS. This number was arrived at from literature searches as well as my own pilot experiments to obtain the optimal amount of virus that would induce an effective immune response without causing serious mortality in low fat diet-fed control mice. Alveolar macrophages respond early upon intranasal challenge, and hence cytokine profiles, phagocytosis etc. were measured at time points 24 h & 48 hours post stimulation. Power analyses were performed using differences in values between the different experimental groups that were inferred to be physiologically relevant from previous experiments and as reported in the literature. For all experiments, the number of animals required in each group was five or less. Hence n = 5 was used for most experiments. For statistical analyses of differences between obese vs lean groups, a two-

tailed Student's t-test was used to determine significance. Analysis of Variance (ANOVA) was also used to compare data across lean and obese groups with or without infection.

Impact of obesity on alveolar macrophage responses to influenza virus infection

Under this aim, I investigated whether AMs contribute to immune responses against influenza virus and to what extent this response is altered by obesity. Alveolar macrophages are crucial for clearing influenza infection. AMs phagocytose influenza virus and influenza-infected cells to eliminate infection. They also secrete pro-inflammatory cytokines such as IFN-α, TNF-α, IL-1β, IL-6, IL-12 and chemokines such as CCL5, CXCL10 and CXCL8, which help to recruit other cells of the immune system such as neutrophils and NK cells to the site of invasion[59,60]. A change in cytokine/chemokine secretion due to obesity can hinder effective clearance of viruses. Under this Aim, I measured the extent to which obesity influences inflammatory and phagocytic responses of AMs.

Impact of obesity on pro-inflammatory vs anti-inflammatory properties of alveolar macrophages:

How does obesity affect the pro-inflammatory vs anti-inflammatory properties of alveolar macrophages? Pro-inflammatory cytokines produced by AMs such as IFN-α, TNF-α, and IL-6 are necessary for clearance of viral infections. AMs not only secrete pro-inflammatory cytokines and chemokines during the initial stages of the immune response, but they also suppress other cell types such as DCs and T cells from causing excessive inflammation, by producing nitric oxide (NO), prostaglandins (PGs), IL-10, transforming growth factor-β (TGF-β) and other cytokines that induce tolerance. They maintain this balance so that invading pathogens are effectively cleared without immune responses becoming overly

detrimental to the host. I wanted to understand whether obesity causes increased susceptibility and worsens outcomes by tampering with this balance. In order to answer this question, AMs were isolated from diet-induced obese and lean mice by bronchoalveolar lavage or density gradient centrifugation of whole lung homogenates (the latter contains both AMs and interstitial macrophages [IMs]). This population of cells were used to quantify levels of IFN-α, TNF-α, IL-6 and IL-10 using Flow Cytometry and ELISA, and comparisons were made across samples. If the AMs from obese mice produce lower amounts of pro-inflammatory cytokines and chemokines as compared with the lean group, a lack of robust immune responses during the initial stages of infection could result in poor clearance and increased susceptibility to the virus. To evaluate macrophage polarization, the AMs were isolated from obese and lean mice by BAL and stained with fluorescent antibodies against F4/80, CD11b, CD11c, MHCII, Gal-3 and CD206. The phenotype of these cells was evaluated using flow cytometry. $F4/80^{+}$ $CD11b^{lo}$ $CD11c^{+}$ $MHCII^{hi}$ $CD206^{-}$ cells are typically M1 macrophages (pro-inflammatory) and $F4/80^{+}$ $CD11b^{lo}$ $CD11c^{+}$ $MHCII^{lo}$ $CD206^{+}$ cells are M2 AMs with a predominantly anti-inflammatory phenotype.

<u>*Are alveolar macrophages in obese mice protective or pathogenic?*</u>

Depletion of alveolar macrophages:

The predominant role AMs play depends on the context of the infection. Hence it is important to know whether obesity alters AM phenotype to impair them, or worse, drive them to be pathogenic. To address this question, lean and diet-induced obese mice were intranasally challenged with clodronate or PBS liposomes. Clodronate liposomes are an effective way to selectively deplete AMs following intranasal administration. AMs

phagocytose the liposomes and the clodronate accumulates in the cell and after reaching intracellular threshold limits, it leads to the AM undergoing apoptosis. After confirming that AMs are eliminated (there were significantly diminished numbers of $CD11b^{lo}$ $CD11c^{+}$ cells in the BAL as observed from flow cytometry), the mice were infected with influenza virus. Mortality and morbidity (as measured by weight loss) scores were measured for different groups, namely: lean with clodronate, lean with PBS, obese with clodronate and obese with PBS, each challenged with either influenza virus or PBS. AMs are essential for protection from influenza as demonstrated by Kim *et al* (2008), Schneider *et al* (2014) and many others. Hence, I expected that depletion of AMs in both groups – lean and obese – will increase mortality, suggesting that AMs are protective against influenza infection. However, I believe that if AMs in obese individuals are functionally impaired, the effects of depletion would be less profound in them in comparison to lean individuals. If AMs in obese individuals are pathogenic, their depletion should improve outcomes upon infection.

Future directions

Adoptive transfer of alveolar macrophages:

If the impairment of AMs due to obesity is the reason for increased susceptibility to influenza, then this might be overcome by transfer of AMs that are functionally robust. To test this possibility, one can adoptively transfer AMs from lean mice to obese mice and vice versa. Then the recipients can be challenged with influenza virus and progression of infection will be evaluated. If AMs in either group have their own intrinsic ability to impart protection or pathogenicity during infection, obese mice receiving AMs from lean mice should be able to clear the pathogen relatively more successfully. However, one major

limitation of such adoptive transfer studies is that the microenvironment in the lungs of obese individuals might interfere with proper AM function even though they have received AMs from healthy donors.

Potential problems & alternative strategies:

Our studies with clodronate are expected to deplete all AM, regardless of their M1 or M2 phenotype. M2 macrophage-specific depletion may be accomplished with mannose-coupled clodronate liposomes can be used to delineate the role of macrophage polarization in infection. An alternative to the clodronate-based depletion strategy is genetically engineered mice that lack lung macrophages. Since the cytokine GM-CSF is essential for development and maturation of AMs, mice that lack its receptor ($Csf2rb^{-/-}$) are deficient in AMs. A limitation of this is that tampering with GM-CSF signaling alters pulmonary immunology apart from depletion of AMs and thus could confound infection outcomes[61]. Another interesting approach is to use CD11c-DTR mice that express diphtheria toxin receptor (DTR) under control of the CD11c promoter. Because mice do not naturally express DTR, only cells expressing CD11c are affected by Diphtheria Toxin (DT). This method followed by intranasal administration of diphtheria toxin (DT) has been used as a tool previously to examine the effect of acute depletion of AMs. The caveats to this approach are that DT could deplete other cells expressing CD11c such as dendritic cells and that neutrophilia in lungs and spleen was observed in CD11c-DTR mice.

Mechanism

Mechanistic aspects of how obesity modulates alveolar macrophage responses to influenza virus infection:

AMs remain largely quiescent when there is no serious pathogenic challenge in the lungs. They also prevent cytotoxic T cells and DCs from mounting a hazardous inflammatory response against innocuous substances such as particles of dust that might enter the lungs during respiration from time to time. AMs should initiate immune response against pathogens but also ensure that the activation of other cells in the lungs does not send the immune system into overdrive. AMs are able to perform these somewhat contrasting functions because they express molecules that are involved in immune activation (pattern recognition receptors such as TLR, and a plethora of pro-inflammatory cytokines) as well as those that can cause immune suppression (molecules like PDL-1 and Gal-3 expressed by AMs inhibit T effector cells). Hence, an impairment of any of these functions could prove to be detrimental to the host. I investigated which of these processes are affected by obesity and how they impair AM function, specifically the impact of obesity on AM phagocytosis, TLR activation and Gal-3 expression.

Obesity and alveolar macrophage phagocytic responses:

How does obesity affect alveolar macrophage phagocytic responses to influenza virus infection?

BAL was performed on lean and obese mice to isolate AM. These AM were then used for phagocytosis assays as follows:

Phagocytosis of fluorescent latex beads: stained with fluorescently tagged AM markers

to label AMs. Then, the fluorescently labeled AMs from lean and obese mice with phagocytosed beads were compared by flow cytometry analysis.

Phagocytosis of apoptotic cells: Briefly, BAL aliquots from lean or obese mice were added to individual wells of 24-well plates and incubated for 2 h at 37°C/5% CO_2. The fluid was then removed by suction and the adherent AM monolayer washed, then re-incubated for 18 h at 37°C/5% CO_2 with fresh culture medium. At this time point, MDCK cells undergoing apoptosis (due to UV exposure from a UV transilluminator) and stained with MitoTracker Red, were added to the wells at a ratio of 10:1. After incubation for 2 h, alveolar macrophages were stained with fluorescently labeled antibodies. After staining, the cells were washed and acquired immediately using a FACSCanto II flow cytometer.

I have obtained preliminary data, which suggest that obesity alters phagocytosis of apoptotic cells (efferocytosis) by AMs *in vitro* without viral challenge. Hence, I believe that AMs from obese mice might show differential phagocytosis of influenza virus as well. This altered phagocytosis may contribute to the dysfunctional immune response. Efferocytosis can often lead to immunosuppression and hence it is possible that pro-inflammatory responses are downregulated in obese mice due to enhanced efferocytosis by AMs. An alternate possibility is that upon viral challenge or a surrogate immune stimulation, efferocytosis is instead impaired in obese mice, leading to worsened disease outcomes.

Obesity and Toll-like Receptor Desensitization:

Are AMs in obese individuals functionally impaired because of Toll-like Receptor (TLR) desensitization? TLRs are important Pattern Recognition Receptors (PRRs) that recognize bacterial and viral components immediately to activate innate immunity. TLRs

expressed on macrophages are crucial for their timely response. Desensitization of TLRs on AMs often leads to enhanced susceptibility to bacterial respiratory pathogens. I hypothesize that such a desensitization mechanism is taking place due to the chronic inflammatory environment that ensues during obesity, which makes obese individuals more susceptible to influenza virus infection. To evaluate AM responses to TLR stimulation in lean and diet-induced obese individuals, I measured AM responses *in vivo* as well as *in vitro* in lean and obese mice. First, AM from lean and diet-induced obese mice were isolated by BAL and then challenged with different TLR ligands – Lipoteichoic Acid (TLR2), Poly (I:C) (TLR3), LPS (TLR4) and Imiquimod (TLR7) or phosphate buffer saline (PBS). Cytokine responses as well surface marker expression of these AMs were also evaluated. TLR-induced cytokine levels were measured using cytokine ELISA or intracellular flow cytometry. The cytokines & chemokines measured include: TNF-α, IFN-α, IL-4, IL-6, IL-10. For *in vivo* studies, lean and diet-induced obese mice were intranasally challenged with the TLR-3 ligand poly (I:C) or phosphate buffer saline (PBS) (for control mice). Poly (I:C) is often used as a surrogate molecule to mimic viral infection because it elicits cytokines responses similar to that is caused by a viral challenge. 24 & 48 hours post stimulation, AMs were isolated from diet-induced obese and lean mice by bronchoalveolar lavage and cytokine levels and surface marker expression was measured.

Future directions:

To address the issue of TLR desensitization *in vivo*, one could use $tlr3^{-/-}$ recipient mice that would receive AM from wild type lean or obese mice. After repopulation by donor AMs, these mice can be intranasally challenged with the TLR3 ligand poly (I:C). Cytokine

responses as well as phagocytosis by AM from these mice can be measured. Since none of the other cells in the recipient animals can respond to poly (I:C) stimulation, one should be able to detect differences in TLR3 activation in AM from lean and obese mice by this method. The use of a $tlr3^{-/-}$ recipient should ensure that change in AM responses and cytokine production is a result of differential TLR signaling in lean and obese AMs themselves and not due to paracrine effects caused by TLR3 activation on other cells.

CHAPTER 3

Results and Discussion

Obesity and infection outcomes

Diet-induced obese mice have increased morbidity and mortality during influenza A virus infection: Similar to published studies, I was able to demonstrate that obesity results in worsened disease outcomes during influenza A virus (IAV) infection. The obese mice had greater weight loss and increased mortality upon infection (Figure 2).

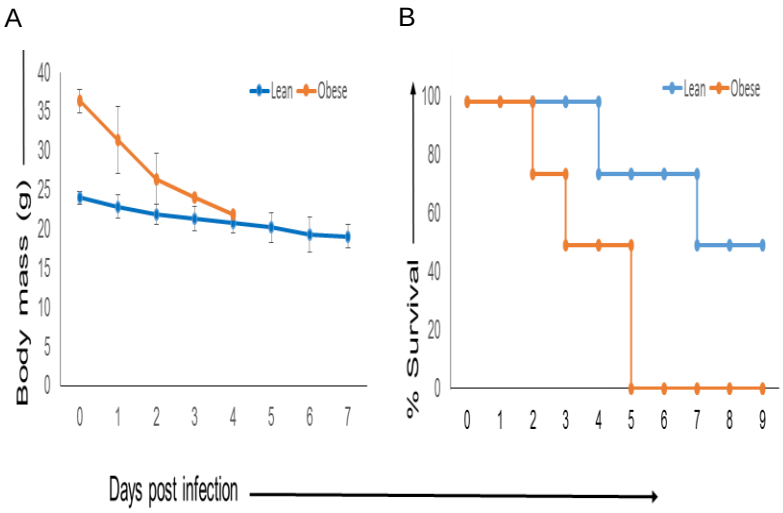

Figure 1: Diet-induced obese mice have increased morbidity and mortality during influenza A virus infection. Lean and Obese mice were infected with 6.5 x 10^3 PFU of IAV and followed for a period of 9 days for body mass (A), and survival (B). Graphs show data from one of two representative experiments. N= 5 or 6 mice

Obesity and alveolar macrophages

<u>*Obesity leads to an increase in overall cell number in the lungs*</u>: In addition to an increase in overall cell number, I found that obesity is also associated with a decrease in alveolar macrophages. I found that Gr-1$^+$ CD11b$^+$ neutrophils increase during obesity. This is

important since changes in immune cell composition in the lungs can directly impact immune response against respiratory pathogens.

Obesity affects alveolar macrophage phenotype: AMs should initiate immune responses against pathogens but also ensure that the activation of other cell types in the lungs does not send the immune system into overdrive[49,50,51,52]. AMs are able to perform these somewhat contrasting functions because they express molecules that are involved in immune activation (pattern recognition receptors such as TLRs, MHC-II and a plethora of pro-inflammatory cytokines) as well as those that can cause immune suppression (molecules like PDL-1 expressed by AMs inhibit T effector cells, CD206 increases phagocytic ability, and Gal-3 induces anti-inflammatory effects)[53,54,55]. Hence impairment of any of these functions could prove to be detrimental to the host. Obesity is known to influence macrophage polarization in the spleen as well as adipose tissue. Hence I investigated if it induces changes in AM polarization as well. For this purpose, I scored the number of AMs expressing CD206, which is an established M2 marker. I also analyzed how the expression of molecules that enhance the inflammatory pathway such as MHC-II, IL-6, and IFN-α, and those that have anti-inflammatory effects such as Gal-3, are affected by obesity.

Obesity leads to an increase in overall cell numbers in the lungs. It is also associated with a decrease in $CD11b^{lo}$ $CD11c^{+}$ alveolar macrophages. I found that $CD206^{+}$ AMs increase during obesity (Figure 3). I also observed that obesity leads to a decrease in AMs that are inflammatory in nature ($MHC\text{-}II^{+}$, $IL\text{-}6^{+}$). This is important since changes in alveolar macrophage polarization can directly impact pulmonary immunology and the immune response against respiratory pathogens.

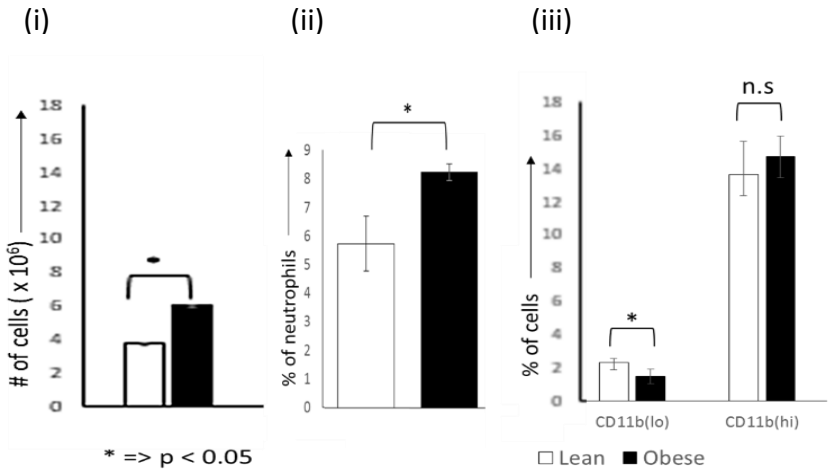

Figure 2. Obesity alters lung cellularity. (i)Total cell number and (ii) frequency of neutrophils ($Gr1^+$ $CD11b^+$) increases during obesity while the fraction of AM ($CD11b^{lo}$ $CD11c^+$) decreases.

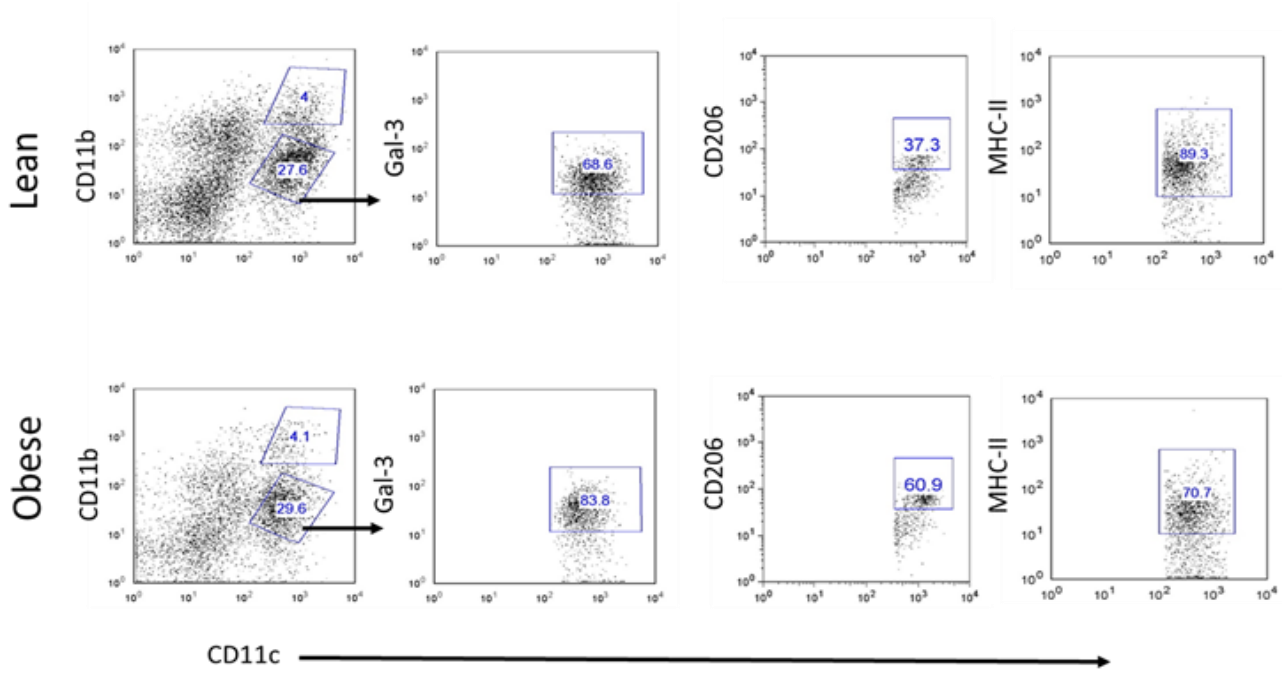

Figure 3. Obesity affects alveolar macrophage phenotype. Flow cytometric analysis of alveolar macrophages ($CD11b^{lo}$ $CD11c^+$) shows that obesity enriches M2-like ($CD206^+$) AMs. A similar increase in AMs expressing Galectin-3, another M2 macrophage marker is observed while the frequency of MHC-II expressing AMs decreases. Flow cytometry dot plots show data from one of two representative experiments. n= 5 mice.

Obesity and alveolar macrophage phagocytic responses

Obesity affects alveolar macrophage phagocytic responses: AM phagocytic responses are important in the clearance of influenza virus. Phagocytosis in general can be a mechanism to eliminate pathogens and modulate immune responses. Previous studies also showed that phagocytosis of apoptotic cells (efferocytosis) during infection by AMs inhibits propagation of the virus. Hence I decided to investigate phagocytosis by AM from lean and DIO mice at basal levels. AM were isolated and their phagocytic ability was measured *in vitro* using fluorescently labeled polystyrene latex beads or apoptotic Madine Darby Canine Kidney (MDCK) cells stained with MitoTracker Red by Flow Cytometry. Interestingly, I found that AM from obese individuals show increased phagocytosis without virus challenge, both beads and apoptotic cells (Figure 4). It is possible that phagocytic responses during infection might be different from steady state conditions. However, efferocytosis often leads to immunosuppression[56,57,58]. Hence whether the increased efferocytic response seen in obese AMs contributes to impaired pro-inflammatory immune responses needs to be investigated. Further, there have been reports of bacteria like *Listeria monocytogenes* using phagocytosis by AM as a means to evade immune surveillance and for propagation. Whether such a mechanism leads to increased spread of the virus and thus contributes to worsened outcomes in obese individuals remains unknown.

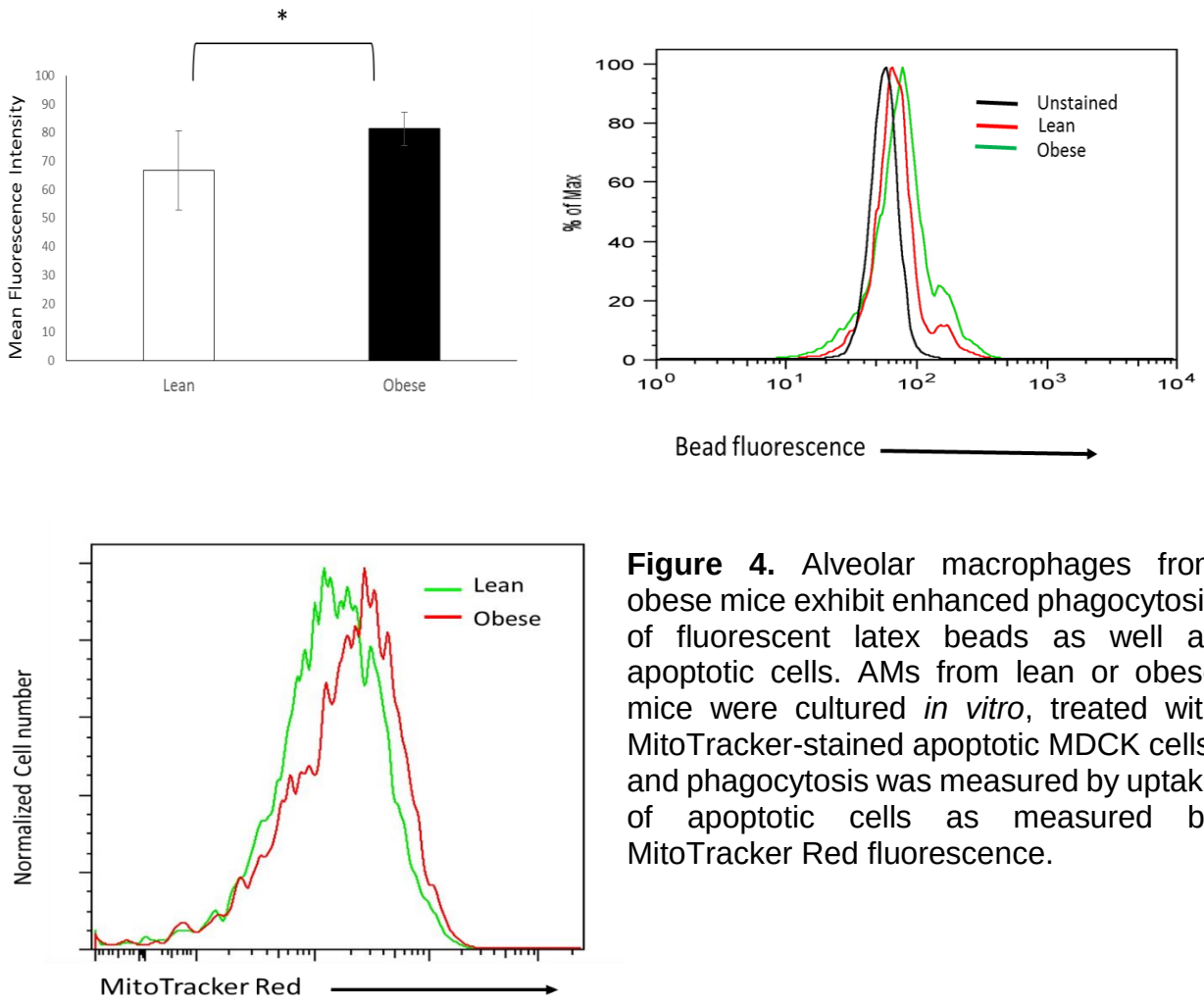

Figure 4. Alveolar macrophages from obese mice exhibit enhanced phagocytosis of fluorescent latex beads as well as apoptotic cells. AMs from lean or obese mice were cultured *in vitro*, treated with MitoTracker-stained apoptotic MDCK cells, and phagocytosis was measured by uptake of apoptotic cells as measured by MitoTracker Red fluorescence.

Increase in efferocytosis by alveolar macrophages correlated with enhanced expression of the protein Tim-3:

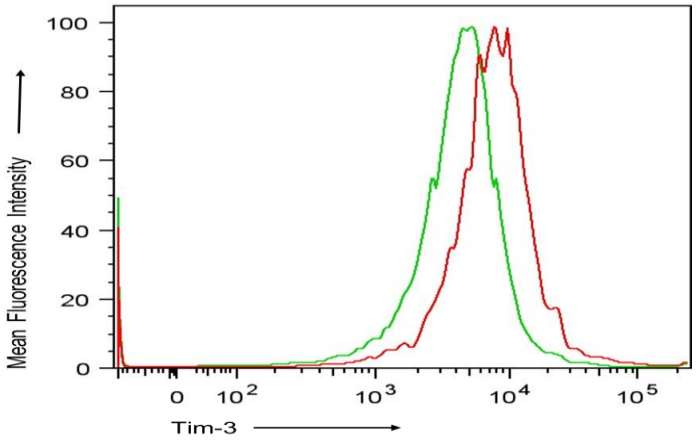

Figure 5. Alveolar macrophages from obese mice show enhanced expression of Tim-3. AM were isolated by BAL, stained with fluorescent anitbodies and expression of Tim-3 was analyzed using flow cytometry. N = 3

Tim-3 is a receptor protein on phagocytic cells that mediates phagocytosis of apoptotic cells. It was first discovered as a negative regulator on Th1 cells. In monocytes, Tim-3 a decrease in Tim-3 expression is associated with a decrease in pro-inflammatory cytokine IL-12. A subsequent blockade of Tim-3 results in decreased levels of Arginase 1 and IL-10, both markers of M2 macrophages, and an increased expression of IL-12 and iNOS which are molecules associated with M1 activation[65]. Tim-3 blockade also leads to an increase in PD-1, which negatively regulates monocyte activation[66]. Overall, such an increase in Tim-3 expression could be at least in part responsible for M2-like polarization and increased efferocytosis exhibited by AM from obese mice.

Impact of obesity on responses mediated by engagement of toll-like receptors

Obesity affects immune responses to in vivo poly(I:C) administration: Poly(I:C) is a synthetic double-stranded RNA (dsRNA) molecule. Since dsRNA is a key indicator of viral replication, it is recognized by the innate immune system to mount an immediate response against the pathogen, a process mediated by pattern recognition receptors such as TLR-3 and RIG-I. This response involves production of type I interferons (IFN-α and IFN-β), antiviral proteins and other pro-inflammatory cytokines. Hence poly(I:C) is often used as a surrogate molecule to mimic viral infection. I administered poly(I:C) to lean and obese mice intranasally and analyzed production of cytokines as well as expression of surface proteins with key immune functions such as Gal-3, MHC-II, CD-206 and CD36.

Preliminary evidence from my experiments indicated that AMs from DIO mice have a greater propensity to assume a less pro-inflammatory phenotype in comparison to their lean counterparts upon TLR stimulation. It also suggested that the cytokine secretion profiles of AMs in obese mice are more anti-inflammatory in nature. If TLR desensitization

is indeed occurring in obese mice, the AMs from obese mice should produce lower amounts of pro-inflammatory cytokines and chemokines as compared to the lean group in response to TLR stimulation.

I found that IL-6 secretion upon poly(I:C) stimulation was hampered in whole lung homogenates and lung macrophages from obese mice (Figure 5A). This was also associated with a decrease in production of the pro-inflammatory cytokines TNF-α and IL-6 by AMs, as observed by intracellular flow cytometry (Figure 5B).

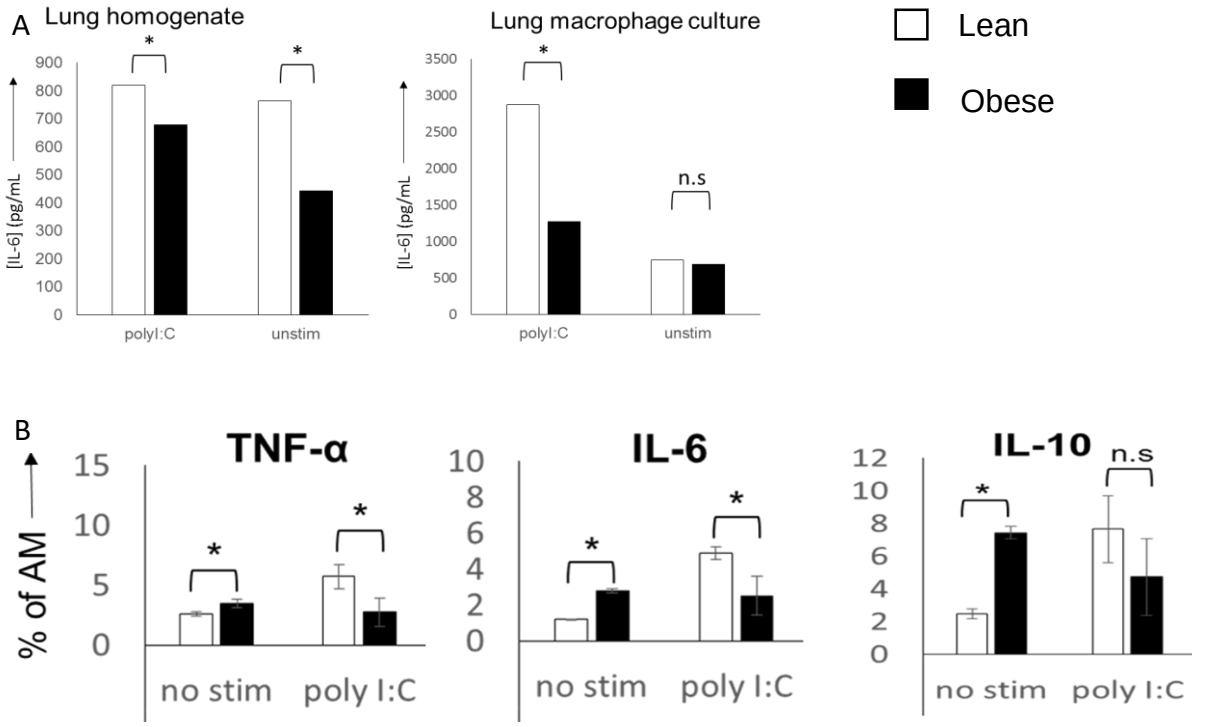

Figure 6. Obesity dampens immune responses to in vivo poly(I:C) stimulation. A. IL-6 production upon poly(I:C) stimulation is hampered in obese mice as evident from cytokine ELISA of (i) lung homogenate or (ii) cell-free supernatants from *in vitro* culture of isolated lung macrophages from mice that were administered poly (I:C) intranasally. B. Intracellular flow cytometry staining shows decreased production of pro-inflammatory cytokines TNF-α and IL-6 by AMs.

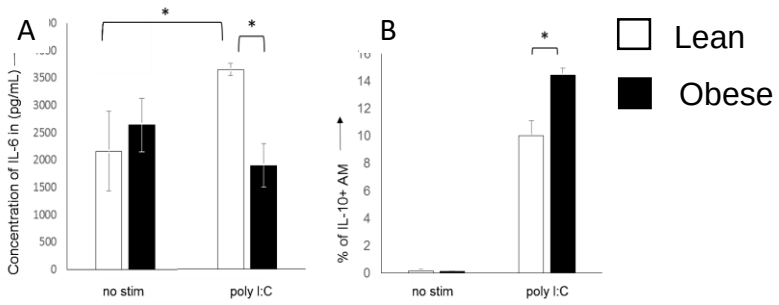

Figure 7. Obesity alters AM responses to TLR3 stimulation. (A) Cytokine ELISA of cell-free supernatants from *in vitro* culture of isolated AM shows impaired IL-6 production in AM from obese mice. (B) Intracellular flow cytometry staining of *in vitro* cultured AMs from obese mice shows higher frequency of IL-10 production upon poly(I:C) stimulation. *p<0.05

These results give credence to the idea that dysfunctional AM responses in obese mice drive changes in pulmonary immunity and contribute to impaired responses against respiratory microbial challenges. They suggest that obesity induces a change in lung macrophage polarization, which causes impaired production of cytokines during infection. To verify whether this change in AM responses and cytokine production was a result of differential TLR signaling in lean and obese AMs themselves and not due to paracrine effects to *in vivo* administration, I isolated AM through BAL, cultured them *in vitro* and analyzed IL-6 and IL-10 production in these cells by ELISA and intracellular flow cytometry respectively. I observed similar results showing decreased IL-6 secretion and an increase in IL-10 production (Figure 6). These findings suggest that impaired TLR signaling caused by the chronic inflammatory environment associated with obesity contributes to impaired AM function and overall immune responses.

Depletion of alveolar macrophages and infection outcomes

Obesity affects alveolar macrophage responses to IAV infection: I depleted AM in lean and obese mice using clodronate liposomes and then infected them with H1N1 PR/8 influenza A virus. Morbidity (based on weight loss) and mortality were assessed in these groups. It has been previously shown that AM are essential for immune responses against

IAV. AMs are essential for protection from influenza as demonstrated by Kim *et al* (2008), Schneider *et al* (2014) and many others. Hence, I expected that depletion of AMs in both groups – lean and obese – will increase mortality, suggesting that AMs are protective against influenza infection. However, if AMs in obese individuals are functionally impaired, the effects of depletion should be less profound in them in comparison to lean individuals. If AMs in obese individuals are pathogenic, their depletion should improve outcomes upon infection. In my experiments as well, I observed similar results with wild type lean mice as observed in previous studies. Lean mice whose AM were depleted using clodronate liposomes had enhanced weight loss and mortality in comparison to lean mice that were administered control PBS liposomes intranasally. Interestingly, obese mice that were administered clodronate liposomes had decreased infection parameters as observed by lesser weight loss and mortality compared to obese mice treated with control liposomes. This suggests that alveolar macrophages in obese mice are dysfunctional (and likely pathogenic), since their depletion in obese mice leads to improved infection outcomes.

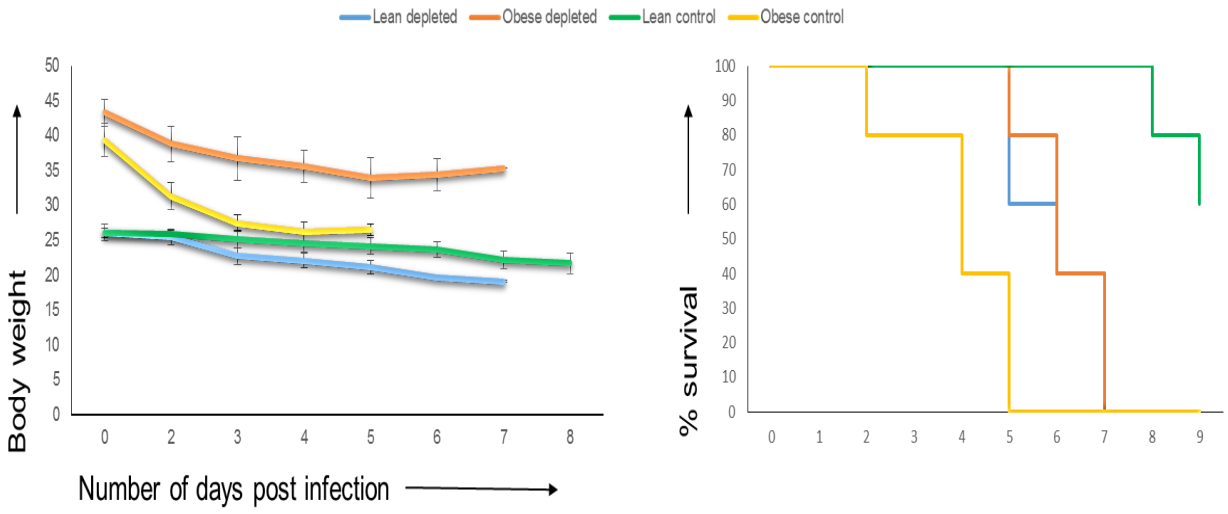

Figure 8. Depletion of alveolar macrophages in obese mice leads to improved infection outcomes during influenza A virus infection. Lean and Obese mice were infected with 6.5 x 10^3 PFU of IAV and followed for a period of 9 days for body mass (A), and survival (B). Graphs show data from one of two representative experiments. N= 5 mice

CHAPTER 4

Summary and Conclusions

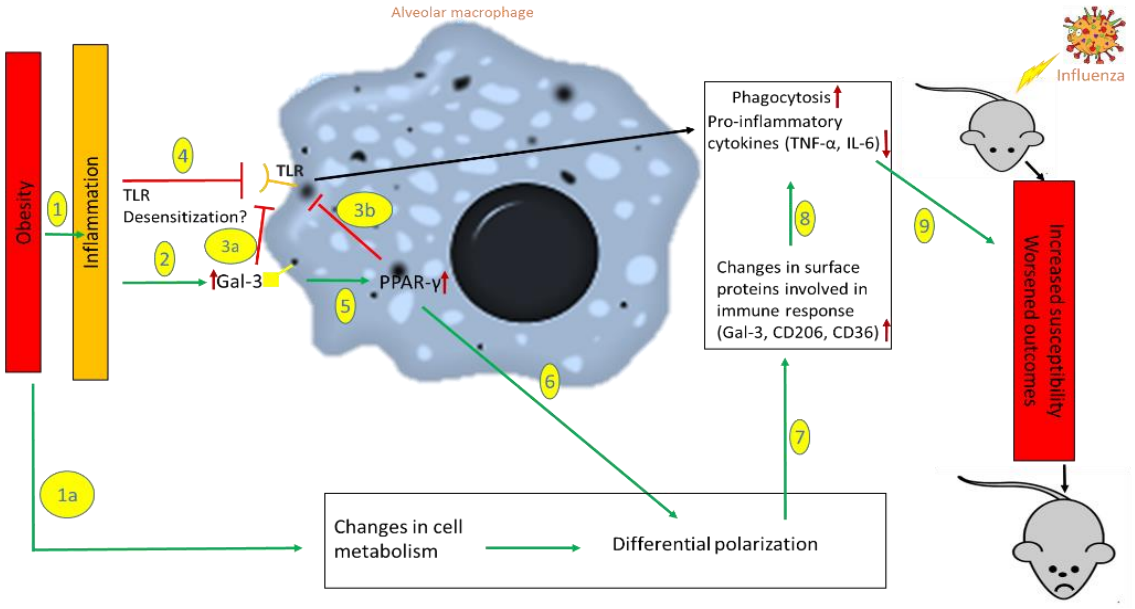

Figure 9. A model for how obesity affects alveolar macrophages and overall immune responses to influenza virus infection.

Obesity increases the risk for influenza A virus infections and worsens outcomes. Alveolar macrophages (AMs) are cells of the immune system that act as primary defenders during respiratory infections including influenza A virus. AMs initiate immune responses against the pathogens but also keep these responses from becoming excessively inflammatory and thus hazardous to the host. Hence I hypothesized that AM dysfunction caused by obesity is an important contributor to the increased susceptibility of obese individuals to influenza virus infection. The main objective of this project was to understand in

mechanistic detail how obesity modulates alveolar responses to influenza virus infection by affecting AM function. To achieve this objective, I 1) determined the extent to which obesity affects AM responses to influenza virus infection, and 2) propose a novel mechanism by which obesity modulates AM responses to influenza virus infection. I have found that obesity induces changes in AM polarization and phagocytic ability, and alterations in the level of surface proteins such as Gal-3, MHC-II, Tim-3, and cytokines such as IL-6, TNF-α, IFN-α and IL-10 in the lung. I have discovered that AMs in obese mice show a more M2-like phenotype (with higher CD206 expression) compared to lean controls. AMs from obese individuals have higher levels of anti-inflammatory cytokines and lower amounts of pro-inflammatory cytokines. This leads to alveolar macrophage dysfunction, potentially rendering them pathogenic and worsens outcomes during influenza A virus infection. This might not only impair immune response by AMs, but may also prevent DCs and T cells from mounting a successful adaptive immune response and thus delay viral clearance, further contributing to detrimental disease outcomes.

Relevance: Influenza is one of the leading causes of infectious disease-related morbidity and mortality in the United States as well as globally. Since obesity has reached pandemic proportions and more than one-third of Americans are obese, understanding how this condition increases the risk of influenza virus infection will help us to develop preventive and therapeutic strategies specific to obese individuals. This project also addresses the impact of cellular metabolism and identification of microbial patterns by specific receptors on immune cells during influenza virus infection in obese and lean individuals. Both of these processes are amenable to manipulation with small molecules and hence their study can lead to potential therapeutic applications.

REFERENCES

1. Punni E, Pula JL, Asslo F, Baddoura W, DeBari VA. Is obesity a risk factor for Clostridium difficile infection? *Obes Res Clin Pract.* (2015) 9(1):50-4. doi:10.1016/j.orcp.2013.12.007.

2. Amar S, Zhou Q, Shaik-Dasthagirisaheb Y, Leeman S. Diet-induced obesity in mice causes changes in immune responses and bone loss manifested by bacterial challenge. *Proc Natl Acad Sci U S A.* (2007) 104(51):20466-71.

3. Richard G, Trivedi N, Belta C, Amar S. Partial restoration of macrophage alteration from diet-induced obesity in response to *Porphyromonas gingivalis* infection. *PLoS One.* (2013) 8(7):e70320. doi:10.1371/journal.pone.0070320.

4. Mancuso P, Gottschalk A, Phare SM, Peters-Golden M, Lukacs NW, Huffnagle GB. Leptin-deficient mice exhibit impaired host defense in Gram-negative pneumonia. *J Immunol.* (2002) 168(8):4018-24.

5. Shrestha SS, Swerdlow DL, Borse RH, Prabhu VS, Finelli L, Atkins CY, Owusu-Edusei K, Bell B, Mead PS, Biggerstaff M, Brammer L, Davidson H, Jernigan D, Jhung MA, Kamimoto LA, Merlin TL, Nowell M, Redd SC, Reed C, Schuchat A, Meltzer MI. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009-April 2010). *Clin Infect Dis.* (2011) 52:S75-82. doi:10.1093/cid/ciq012.

6. Dawood FS, Iuliano AD, Reed C, Meltzer MI, Shay DK, Cheng PY, Bandaranayake D, Breiman RF, Brooks WA, Buchy P, Feikin DR, Fowler KB, Gordon A, Hien NT, Horby P, Huang QS, Katz MA, Krishnan A, Lal R, Montgomery JM, Mølbak K, Pebody R, Presanis AM, Razuri H, Steens A, Tinoco YO, Wallinga J, Yu H, Vong S, Bresee J, Widdowson MA. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. *Lancet Infect Dis.* (2012) 12(9):687-95. doi: 10.1016/S1473-3099(12)70121 -4.

7. Smith AG, Sheridan PA, Tseng RJ, Sheridan JF, Beck MA. Selective impairment in dendritic cell function and altered antigen-specific CD8+ T-cell responses in diet-induced obese mice infected with influenza virus. *Immunology.* (2009) 126(2):268-79. doi: 10.1111/j.1365-2567.2008.02895.x.

8. O'Shea D, Corrigan M, Dunne MR, Jackson R, Woods C, Gaoatswe G, Moynagh PN, O'Connell J, Hogan AE. Changes in human dendritic cell number and function in severe obesity may contribute to increased susceptibility to viral infection. *Int J Obes.* (2013) 37(11):1510-3. doi: 10.1038/ijo.2013.16.

9. Karlsson EA, Sheridan PA, Beck MA. Diet-induced obesity in mice reduces the maintenance of influenza-specific CD8+ memory T cells. *J Nutr.* (2010) 140(9):1691 -7. doi: 10.3945/jn.110.123653.

10. Karlsson EA, Sheridan PA, Beck MA. Diet-induced obesity impairs the T cell memory response to influenza virus infection. *J Immunol.* (2010) 184(6):3127-33. doi:10.4049/jimmunol.0903220.

11. Khan SH, Hemann EA, Legge KL, Norian LA, Badovinac VP. Diet-induced obesity does not impact the generation and maintenance of primary memory CD8 T cells. *J Immunol.* (2014) 193(12):5873-82. doi: 10.4049/jimmunol.1401685.

12. Milner JJ, Sheridan PA, Karlsson EA, Schultz-Cherry S, Shi Q, Beck MA. Diet-induced obese mice exhibit altered heterologous immunity during a secondary 2009 pandemic H1N1 infection. *J Immunol.* (2013) 191(5):2474-85. doi: 10.4049/jimmunol.1202429.

13. Zhang AJ, To KK, Li C, Lau CC, Poon VK, Chan CC, Zheng BJ, Hung IF, Lam KS, Xu A, Yuen KY. Leptin mediates the pathogenesis of severe 2009 pandemic influenza A(H1N1) infection associated with cytokine dysregulation in mice with diet-induced obesity. *J Infect Dis.* (2013) 207(8):1270-80. doi:10.1093/infdis/jit031.

14. Wieland CW, Florquin S, Chan ED, Leemans JC, Weijer S, Verbon A, Fantuzzi G, van der Poll T. Pulmonary *Mycobacterium tuberculosis* infection in leptin-deficient ob/ob mice. *Int Immunol.* (2005) 17(11):1399-408.

15. Tsatsanis C, Margioris AN, Kontoyiannis DP. Association between H1N1 infection severity and obesity-adiponectin as a potential etiologic factor. *J Infect Dis.* (2010) 202(3):459-60. doi:
10.1086/653842.

16. Milner JJ, Rebeles J, Dhungana S, Stewart DA, Sumner SC, Meyers MH, Mancuso P, Beck MA. Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice. *J Immunol.* (2015) 194(10):4846-59. doi: 10.4049/jimmunol.1402295.

17. Nieman DC, Henson DA, Nehlsen-Cannarella SL, Ekkens M, Utter AC, Butterworth DE, Fagoaga OR. Influence of obesity on immune function. *J Am Diet Assoc.* (1999) 99(3):294-9.

18. Schneider C, Nobs SP, Heer AK, Kurrer M, Klinke G, van Rooijen N, Vogel J, Kopf M. Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. *PLoS Pathog.* (2014)10(4):e1004053. doi: 10.1371/journal.ppat.1004053.

19. Kim HM, Lee YW, Lee KJ, Kim HS, Cho SW, van Rooijen N, Guan Y, Seo SH. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. *J Virol.* (2008) 82(9):4265-74. doi:10.1128/JVI.02602-07.

20. Tracey VV, De NC, Harper JR. Obesity and respiratory infection in infants and young children. *Br Med J.* (1971) 1(5739):16-8.

21. Falagas ME, Kompoti M. Obesity and infection. *Lancet Infect Dis.* (2006) 6(7):438-46.

22. Morgan OW, Bramley A, Fowlkes A, Freedman DS, Taylor TH, Gargiullo P, Belay B, Jain S, Cox C, Kamimoto L, Fiore A, Finelli L, Olsen SJ, Fry AM. Morbid obesity as a risk factor for hospitalization and death due to 2009 pandemic influenza A(H1N1) disease. *PLoS One.* (2010) 5(3):e9694. doi:10.1371/journal.pone.0009694.

23. Hagau N, Slavcovici A, Gonganau DN, Oltean S, Dirzu DS, Brezoszki ES, Maxim M, Ciuce C, Mlesnite M, Gavrus RL, Laslo C, Hagau R, Petrescu M, Studnicska DM. Clinical aspects and cytokine response in severe H1N1 influenza A virus infection. *Crit Care*. (2010) 14(6):R203. doi: 10.1186/cc9324.

24. Garcia MN, Philpott DC, Murray KO, Ontiveros A, Revell PA, Chandramohan L, Munoz FM. Clinical predictors of disease severity during the 2009-2010 A(HIN1) influenza virus pandemic in a paediatric population. *Epidemiol Infect.* (2015) 1 -11.

25. Smith AG, Sheridan PA, Harp JB, Beck MA. Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. *J Nutr.* (2007) 137(5):1236-43.

26. Sheridan PA, Paich HA, Handy J, Karlsson EA, Hudgens MG, Sammon AB, Holland LA, Weir S, Noah TL, Beck MA. Obesity is associated with impaired immune response to influenza vaccination in humans. *Int J Obes.* (2012) 36(8):1072-7. doi: 10.1038/ijo.2011.208.

27. Babaev VR, Yancey PG, Ryzhov SV, Kon V, Breyer MD, Magnuson MA, Fazio S, Linton MF. Conditional knockout of macrophage PPARγ increases atherosclerosis in C57BL/6 and low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. (2005) 25:1647–1653.

28. Baker AD, Malur A, Barna BP, Ghosh S, Kavuru MS, Malur AG, Thomassen MJ. Targeted PPAR{γ} deficiency in alveolar macrophages disrupts surfactant catabolism. *J Lipid Res.* (2010) 51:1325–1331.

29. Malur A, McCoy AJ, Arce S, Barna BP, Kavuru MS, Malur AG, Thomassen MJ. Deletion of PPAR γ in alveolar macrophages is associated with a Th-1 pulmonary inflammatory response. *J Immunol.* (2009) 182, 5816 5822.

30. Baker AD, Malur A, Barna BP, Kavuru MS, Malur AG, Thomassen MJ. PPARγ regulates the expression of cholesterol metabolism genes in alveolar macrophages. *Biochem Biophys Res Commun*. (2010) 393:682–687.

31. Schneider C, Nobs SP, Kurrer M, Rehrauer H, Thiele C, Kopf M. Induction of the nuclear receptor PPAR-γ by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages. *Nat Immunol.* (2014) (11):1026-37. doi: 10.1038/ni.3005.

32. Baek JH, Kim SJ, Kang HG, Lee HW, Kim JH, Hwang KA, Song J, Chun KH. Galectin-3 activates PPARγ and supports white adipose tissue formation and high-fat diet-induced obesity. *Endocrinology*. (2015) 156(1):147-56. doi: 10.1210/en.2014-1374.

33. MacKinnon AC, Farnworth SL, Hodkinson PS, Henderson NC, Atkinson KM, Leffler H, Nilsson UJ, Haslett C, Forbes SJ, Sethi T. Regulation of alternative macrophage activation by galectin-3. *J Immunol.* (2008) 180(4):2650-8.

34. Li Y, Komai-Koma M, Gilchrist DS, Hsu DK, Liu FT, Springall T, Xu D. Galectin-3 is a negative regulator of lipopolysaccharide-mediated inflammation. *J Immunol.* (2008) 181(4):2781-9.

35. Breuilh L, Vanhoutte F, Fontaine J, van Stijn CM, Tillie-Leblond I, Capron M, Faveeuw C, Jouault T, van Die I, Gosset P, Trottein F. Galectin-3 modulates immune and inflammatory responses during helminthic infection: impact of galectin-3 deficiency on the functions of dendritic cells. *Infect Immun.* (2007) 75(11):5148-57.

36. Appel S, Mirakaj V, Bringmann A, Weck MM, Grünebach F, Brossart P. PPAR-gamma agonists inhibit toll-like receptor-mediated activation of dendritic cells via the MAP kinase and NF-kappaB pathways. *Blood*. (2005) 106(12):3888-94.

37. Zhao W, Wang L, Zhang M, Wang P, Zhang L, Yuan C, Qi J, Qiao Y, Kuo PC, Gao C. Peroxisome proliferator-activated receptor gamma negatively regulates IFN-beta production in Toll-like receptor (TLR) 3- and TLR4-stimulated macrophages by preventing interferon regulatory factor 3 binding to the IFN-beta promoter. *J Biol Chem*. (2011) 286(7):5519-28. doi: 10.1074/jbc.M110.149823.

38. Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M, Lawrence T, van Rijt LS, Lambrecht BN, Sirard JC, Hussell T. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. *J Exp Med.* (2008) 205(2):323-9. doi: 10.1084/jem.20070891 .

39. Prieur X, Mok CY, Velagapudi VR, Núñez V, Fuentes L, Montaner D, Ishikawa K, Camacho A, Barbarroja N, O'Rahilly S, Sethi JK, Dopazo J, Orešič M, Ricote M, Vidal-Puig A. Differential lipid partitioning between adipocytes and tissue macrophages modulates macrophage lipotoxicity and M2/M1 polarization in obese mice. *Diabetes.* (2011) 60(3):797-809. doi: 10.2337/db10-0705.

40. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA. Macrophage activation and polarization:

nomenclature and experimental guidelines. *Immunity*. (2014) 41(1):14-20. doi: 10.1016/j.immuni.2014.06.008.

41. Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol*. (2014) 14(2):81 -93. doi: 10.1038/nri3600.

42. Huang SC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, Beatty W, Love-Gregory L, Lam WY, O'Neill CM, Yan C, Du H, Abumrad AN, Urban Jr. JF, Artyomov MN, Pearce EL & Pearce EJ. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. Nat Immunol. (2014) 15(9):846-55. doi: 10.1038/ni.2956.

43. O'Sullivan D, van der Windt GJ, Huang SC, Curtis JD, Chang CH, Buck MD, Qiu J, Smith AM, Lam WY, DiPlato LM, Hsu FF, Birnbaum MJ, Pearce EJ, Pearce EL. Memory CD8(+) T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. *Immunity*. (2014) 41(1):75-88. doi:10.1016/j.immuni.2014.06.005.

44. Baritussio A, Enzi G, Inelmen EM, Schiavon M, de Biasi F, Allegra L, Ursini F, Baldo G. Altered surfactant synthesis and function in rats with diet-induced hyperlipidemia. *Metabolism* (1980) 29:503e10.

45. Madenspacher JH, Draper DW, Smoak KA, Li H, Griffiths GL, Suratt BT, Wilson MD, Rudel LL, Fessler MB. Dyslipidemia induces opposing effects on intrapulmonary and extrapulmonary host defense through divergent TLR response phenotypes. *J Immunol* (2010) 185:1660 – 9

46. Lee JY, Zhao L, Youn HS, Weatherill AR, Tapping R, Feng L et al. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. *J Biol Chem* (2004) 279:16971 –16979.

47. Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999– 2008. *JAMA* (2010) 303(3):235 – 41.

48. Wu L, Parekh VV, Hsiao J, Kitamura D, Van Kaer L. Spleen supports a pool of innate-like B cells in white adipose tissue that protects against obesity-associated insulin resistance. Proc Natl Acad Sci U S A. (2014) 111(43):E4638-47. doi: 10.1073/pnas.1324052111.

49. Hashimoto Y, Moki T, Takizawa T, Shiratsuchi A, Nakanishi Y. Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. *J Immunol.* (2007) 15;178(4):2448-57.

50. Ghoneim HE, Thomas PG, McCullers JA. Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections. *J Immunol.* (2013) 191(3):1250-9. doi:10.4049/jimmunol.1300014.

51. Aggarwal NR, Tsushima K, Eto Y, Tripathi A, Mandke P, Mock JR, Garibaldi BT, Singer BD, Sidhaye VK, Horton MR, King LS, D'Alessio FR. Immunological priming requires regulatory T cells and IL-10-producing macrophages to accelerate resolution from severe lung inflammation. *J Immunol*. (2014) 192(9):4453-64. doi: 10.4049/jimmunol.1400146.

52. Pribul PK, Harker J, Wang B, Wang H, Tregoning JS, Schwarze J, Openshaw PJ. Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. *J Virol.* (2008) 82(9):4441 -8. doi: 10.1128/JVI.02541 -07.

53. Huber S, Hoffmann R, Muskens F, Voehringer D. Alternatively activated macrophages inhibit T-cell proliferation by Stat6-dependent expression of PD-L2. Blood. (2010) 28;116(17):3311 -20. doi:10.1182/blood-2010-02-271981.

54. Pechkovsky DV, Prasse A, Kollert F, Engel KM, Dentler J, Luttmann W, Friedrich K, Müller-Quernheim J, Zissel G. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. Clin Immunol. (2010) 137(1):89-101. doi:10.1016/j.clim.2010.06.017.

55. Dietze J, Böcking C, Heverhagen JT, Voelker MN, Renz H. Obesity lowers the threshold of allergic sensitization and augments airway eosinophilia in a mouse model of asthma. Allergy. (2012) 67(12):1519-29. doi: 10.1111/all.12031.

56. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol.* (2002) 2(12):965-75.

57. Czuczman MA, Fattouh R, van Rijn JM, Canadien V, Osborne S, Muise AM, Kuchroo VK, Higgins DE, Brumell JH. Listeria monocytogenes exploits efferocytosis to promote cell-to-cell spread. *Nature*. (2014) 509(7499):230-4.

58. Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest.* (2001) 108(7):957-62.

59. Hastings AK, Erickson JJ, Schuster JE, Boyd KL, Tollefson SJ, Johnson M, Gilchuk P, Joyce S, Williams JV. Role of type I interferon signaling in human metapneumovirus pathogenesis and control of viral replication. *J Virol.* (2015) 89(8):4405-20. doi: 10.1128/JVI.03275-14.

60. Yasui F, Kohara M, Kitabatake M, Nishiwaki T, Fujii H, Tateno C, Yoneda M, Morita K, Matsushima K, Koyasu S, Kai C. Phagocytic cells contribute to the antibody-mediated elimination of pulmonaryinfected SARS coronavirus. *Virology*. (2014) 454-455:157-68. doi: 10.1016/j.virol.2014.02.005.

61. Huang FF, Barnes PF, Feng Y, Donis R, Chroneos ZC, Idell S, Allen T, Perez DR, Whitsett JA, Dunussi-Joannopoulos K, Shams H. GM-CSF in the lung protects against

lethal influenza infection. *Am J Respir Crit Care Med.* (2011)184(2):259-68. doi: 10.1164/rccm.201012-2036OC.

62. Duan M, Li WC, Vlahos R, Maxwell MJ, Anderson GP, Hibbs ML. Distinct macrophage subpopulations characterize acute infection and chronic inflammatory lung disease. *J Immunol.* (2012) 189(2):946-55. doi: 10.4049/jimmunol.1200660.

63. Zaynagetdinov R, Sherrill TP, Kendall PL, Segal BH, Weller KP, Tighe RM, Blackwell TS. Identification of myeloid cell subsets in murine lungs using flow cytometry. *Am J Respir Cell Mol Biol*. (2013) 49(2):180-9. doi: 10.1165/rcmb.2012-0366MA.

64. Guth AM, Janssen WJ, Bosio CM, Crouch EC, Henson PM, Dow SW. Lung environment determines unique phenotype of alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol*. (2009) 296(6):L936-46. doi: 10.1152/ajplung.90625.2008.

65. Hou N, Piao X, Liu S, Wu C, Chen Q. Tim-3 induces Th2-biased immunity and alternative macrophage activation during *Schistosoma japonicum* infection. *Infect Immun.* (2015) 83(8):3074-82. doi: 10.1128/IAI.00517-15.

66. Brown KE, Freeman GJ, Wherry EJ, Sharpe AH. Role of PD-1 in regulating acute infections. *Curr. Opin. Immunol.* (2010) 22:397–401

67. Armani A, Mammi C, Marzolla V, Calanchini M, Antelmi A, Rosano GMC, Fabbri A, Caprio M. Cellular Models for Understanding Adipogenesis, Adipose Dysfunction, and Obesity. *J Cell Biochem.* (2010) 110:564–572.

68. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. *Annu Rev. Immunol.* (2011) 29:415–445