Immunomodulation of influenza infection by *Echinacea* and obesity

by

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ABSTRACT

Echinacea is a medicinal plant that has a history of use for the treatment of respiratory tract infections. More recently there has been a renewed interest in alternative therapies including phytomedicinal preparations of *Echinacea*. Some important issues that remain to be addressed regarding the use of *Echinacea* therapeutically include plant species specific alterations of immune response and the effect of different preparation techniques (water as compared to ethanol) on immune response to viral infection. Results from multiple studies in mice, in cell culture, and in clinical trials in humans have suggested that water preparations provide a stimulatory effect on innate immunity by enhancing macrophage activation, whereas ethanol extracts decrease inflammation associated cytokines/chemokines. However, the mechanisms by which different plant species enhance immunity against influenza infection and improve disease outcome remain to be clearly elucidated. In this dissertation, both water and ethanol extracts of three species of *Echinacea* were used to assess alterations in the immune response to influenza A/PR/8/34 virus infection. Studies focused on the impact of *Echinacea* treatment during influenza infection on 1) *in vitro* models of viral load and immune responses in the respiratory tract epithelial cells and immune cells, 2) combination treatment of water and ethanol extracts delivered at specific phases of influenza infection and the impact on "traditional" immune measures as well as a bioinformatics approach to lung gene regulation using the Database for Annotation Visualization and integrated Discovery (DAVID), and 3) immune response to influenza in obese mice treated with *Echinacea* water extract. The primary topic of this dissertation was the effect of *Echinacea* on immunity. However, there was also an emphasis on the topic of obesity

and immunity. The results from many studies have shown diet-induced obesity related immune dysfunction in the context of the immune response to influenza virus and vaccination. In this dissertation, the effect of *Echinacea* treatment on obesity associated immune impairments to influenza infection was also evaluated.

The first *Echinacea* immunity study (Chapter II) examined the effect of three species of *Echinacea* for anti-viral effects against influenza A/PR/8/34 virus, as well as the effect of the same three species of *Echinacea* on the lung epithelial immune response to influenza A/PR/8/34 virus, and on immune cells isolated from the spleens of infected BALB/c mice stimulated with A/PR/8/34 *in vitro*. The findings from this study showed that *E. purpurea* water extracts demonstrated modest anti-viral activity at a low or high dose, whereas the ethanol extracts of *E. purpurea*, *E. angustifolia*, and *E. paradoxa var. paradoxa* demonstrated modest anti-viral activity only at a high dose. Studies have shown that polysaccharides (hydrophilic) and alkamides (lipophilic) isolated from *Echinacea* extracts have anti-viral activity *in vitro*. Both water and ethanol extracts enhanced the lung epithelial and immune cell response through modulation of cytokine/chemokine responses that may be involved in recruiting immune cells to the site of infection (such as monocyte inflammatory protein 1 alpha: MIP-1α, monocyte inflammatory protein 1 beta: MIP-1β, interleukin 8: IL8, Eotaxin, granulocyte macrophage colony stimulating factor: GM-CSF) and differentiation of T cells into subtypes (interferon gamma: IFN-γ, interleukin 2: IL2, interleukin 4: IL4, interleukin 5: IL5, interleukin 17: IL17), as well as, decreased inflammatory cytokines (interleukin 6: IL-6 and tumor necrosis factor alpha: TNFα) in the presence of influenza A/PR/8/34 infection. Based on these findings, *Echinacea* extracts may enhance the immune

response to influenza infection by decreasing viral load, regulating cytokines/chemokines involved with cellular response to influenza, and limiting influenza associated inflammation

A subsequent study (Chapter III) evaluated the effect of using a combination of *Echinacea* extracts delivered at specific times during the infection in BALB/c mice infected with influenza A/PR/8/34 virus using *Echinacea angustifolia,* and *Echinacea paradoxa var. paradoxa*. These species were chosen based on previous unpublished work and as an extension of the *in vitro* work completed in Chapter II. Also, *E. angustifolia* is a species that is commonly found in commercial preparations, whereas, *E. paradoxa var. paradoxa* has not been studied in the context of influenza infection. In this study, the effect of combination treatment on the lung viral load, lung cell populations, lung lesion scores, and lung cytokine/chemokine production were evaluated. We also utilized DAVID bioinformatics to determine the effect of *Echinacea* treatment in the lungs of mice infected with influenza at day 8 and 9 post infection. We demonstrated that the combination treatment had no significant effect on illness severity as measured by body weight, and no effect on lung lesion scores or lung cell populations. However, there was an anti-inflammatory with respect to the concentration of several inflammatory cytokines and chemokines in the lungs observed in mice treated with the *E. angustifolia* water extract followed by the *E. angustifolia* EtOH extract. Gene expression results are suggestive of decreased chemokine expression as well. The gene profile also suggested that there was an activation of immune cells and a down regulation of genes involved in wound repair. Anti-influenza specific antibody was increased in the mice that received the combination of *E. angustifolia* water followed by *E. angustifolia* ethanol. The overall

results showed that the immuno-modulatory effects are determined by *Echinacea* species utilized. The data in this study suggest the possibility of enhancing the innate and cellular immune response particularly through gene regulation of macrophage phagocytosis, lymphocyte activation and differentiation, along with increased antibody production by utilizing an *Echinacea* water extract at the early phase of infection. Also, the data in this study support a decrease in pro-inflammatory cytokines, decreased chemokine responsible for neutrophil recruitment, and down-regulation of genes associated with the wound response and neutrophil recruitment when *Echinacea* ethanol extract was administered during the late phase of influenza infection. The conclusions from the combination study suggest that *Echinacea* combination treatment can have a beneficial effect on immune cell activation and decreased inflammation in the lung dependent on the *Echinacea* species.

Finally, in Chapter IV of this dissertation, the effect of *Echinacea* on the immune response to Influenza A/PR/8/34 virus (IAV) infection in diet-induced obese mice was evaluated. Obesity resulting from a high fat diet, genetic deficiencies, and sedentary lifestyles has been shown to have a negative impact on the immune system resulting in immune dysregulation. In the 2009 H1N1 pandemic, obesity was found to be a risk factor for increased severity of infections. The purpose of the last study in this dissertation was to determine whether *Echinacea* might improve immune responsiveness to IAV in diet-induced obese mice (receiving a high fat diet for eight weeks) compared to lean mice (receiving a low fat diet for eight weeks) through immune enhancement of the innate and adaptive immune response. Diet induced obesity has been associated with increased pro-inflammatory cytokines in the uninfected state (interleukin 1 beta: IL-1β,

IL-6, TNF-α) and decreased cellular recruitment to the lung during infection, (decreased dendritic cells, natural killer cells, influenza specific CD8 cells, decreased IFN-γ production). The obesity effects seen in the diet induced obesity model used in this study correlated with the findings of previous studies. *Echinacea* treatment increased alveolar macrophage percentage, and decreased CD8 in the lungs of obese mice, and altered the IFN-γ production in spleen cells. Overall the findings suggest that *Echinacea* was not able to restore the obesity associated immune dysfunction.

CHAPTER I

GENERAL INTRODUCTION

Organization of the Dissertation:

This dissertation consists of manuscripts that are being prepared for submission to peer-reviewed journals. The dissertation contains a total of five chapters including a general introduction, three research papers, and a general conclusion that discusses the overall findings from the dissertation, followed by acknowledgements. The references for each chapter, including Chapter I (Literature Review), are listed at the end of each chapter with figures following the references directly. The General Introduction (Chapter I) to the dissertation includes the impact of influenza on public health and a brief introduction to *Echinacea* as a remedy for influenza infection along with a brief description of the research objective of the dissertation. The Literature Review provides background information on the lifecycle of influenza viruses, followed by a review of the host immune response to the virus, along with a review of *Echinacea* and the known effects of *Echinacea* on the immune system. Finally, a brief summary of diet-induced obesity associated immune impairments closes the review of literature. Chapter II is focused primarily on evaluating immune changes with *Echinacea* treatment during influenza infection *in vitro*. Chapter III is focused on utilizing a combination of *Echinacea* extracts delivered at specific phases of infection to modulate the immune response to influenza during the early and late phases of infection. Chapter IV specifically addresses diet induced obesity associated immune dysfunction in influenza infection and whether *Echinacea* treatment can correct those immune impairments

This dissertation contains the experimental data and results obtained by the author during her Ph.D. study under the supervision of her major professor Dr. Marian L. Kohut at Iowa State University, Immunobiology Interdepartmental Program.

Introduction to the Dissertation:

Infection with influenza virus remains a significant cause of morbidity and mortality. Approximately 100,000 hospitalizations 36,000 deaths occur every influenza season in the US alone [1]. Seasonal vaccination is currently the only prevention for infection with influenza virus. Several anti-viral medications are available for treatment of severe cases of influenza; however, there is a risk of viral mutation leading to resistance for the current treatments. With a growing trend towards the use of "natural" remedies for infections in general, *Echinacea* has emerged as a popular treatment for upper respiratory infections. Native to North America, *Echinacea* was originally used by Native Americans. In the 1900's, *Echinacea* was first used to combat colds and flu-like symptoms and was introduced into Europe [2]. In recent years, it has re-emerged as a popular alternative treatment for influenza infections in the US and is available in commercial preparations such as Airborne®, EpiCore® and Echinaforce®. A lack of standardization in botanical remedies and proprietary blends of commercial preparations adds to the variability of efficacy achieved with *Echinacea*. Studies have shown that *Echinacea* is effective at stimulating the innate immune system, particularly macrophages, natural killer (NK) and dendritic cells (DC) and decreasing inflammation associated cytokines. These effects on the immune system vary by *Echinacea* species utilized, preparation techniques, and plant part used for the extraction. Very few studies have investigated the potential benefit of *Echinacea* during influenza infection to determine whether different species of *Echinacea* have different immunomodulatory

effects. Given that the active constituents of *Echinacea* may vary by species and type of extract preparation, using different species of *Echinacea*, and/or a combination of different types of extract preparations may result in optimal benefits for host defense against influenza. In this dissertation, this type of combination approach was used a novel approach to balancing the immune stimulatory properties of *Echinacea* with the anti-inflammatory properties. In the 2009 H1N1 influenza pandemic, obesity emerged as a risk factor for severe influenza infections with increased morbidity and mortality associated with the obese condition. Obesity (resulting from dietary, genetic, and lifestyle factors) is becoming a national epidemic of its own with 35.6% of adults in the US classified as obese 2009-2010 [3]. Obesity has been shown to have immune related dysfunctions, such as increased pro-inflammatory cytokines and decreased cellular function, and really highlights the importance of nutrition in the function of a proper immune response. The effect of *Echinacea* on influenza in the obese population and whether it has benefits for immune enhancement in diet induced obesity has not been previously investigated.

Literature Review

Influenza structure and pathogenesis

Influenza is a negative sense, single strand RNA virus in the family *Orthomyxoviridae*. Influenza remains a respiratory pathogen causing significant yearly morbidity and mortality in the US and worldwide, hospitalizing over 200,000 individuals and causing approximately 36,000 fatalities per season (November to April) in the US alone [1]. Seasonal influenza infections commonly impact the older populations (over

65) and the very young (less than 3). It has been suggested that pandemic infections typically impact the middle age population more readily (age 20-40). Influenza has several different strains: A, B, and C with influenza A being the most pathogenic. Several pandemics of influenza A have been recorded including 1889, 1918, 1957, 1968, and 2009 with the pandemic of 1918 being the most deadly pandemic of influenza on record. Influenza B and C have not been associated with pandemics to date.

The basic structure of the virus includes a hemagglutination protein (HA), neuriminadase protein (NA) and matrix 2 protein (M2). There are sixteen known HA proteins and nine known NA. HA is responsible for attachment of the virus to the host. NA is speculated to be involved in the release of the virus for purposes of transmission to a new host or new cell. The virion RNA is made of eight gene segments allowing for large diversity in virus strains. Natural hosts for influenza A are wild birds although it is able to infect a variety of host mammals [4,5]. The ability to adapt and infect new hosts (zoonotic transmission) solidifies influenza as a constant pathogenic threat to humans. As the virus infects new hosts (host switching), the recombination with other influenza viruses present in the new host may occur. This recombination allows for new combinations of HA and NA proteins to emerge in a process called reassortment. For the virus to be transmitted further to a human host, several changes must occur. It has been hypothesized that a single amino acid mutation allows for the HA to switch from a sialic acid preference of α2-3Gal linkage found in water fowl to a sialic acid preference of α2-6Gal linkage found in the respiratory tract of humans [5-7]. The M2 protein forms ion channels in the host cell to help facilitate viral replication through pH neutralization of

Golgi bodies [8]. The HA, NA, and M2 proteins are the three dominant proteins of the viral envelope membrane to which host antibodies are formed.

The frequent mutation of influenza viruses through "antigenic drifts" and "antigenic shifts" confer immune evasive properties to the virus. Because influenza A viruses are RNA based, they have no editing machinery to fix point mutations during the replication process. This lack of machinery coupled with a high replication rate causes point mutations to accumulate in the virus leading to an "antigenic drift". Antigenic drift allows the virus to evade the immune system due to changes in the virus that disables the immune system from recognizing the appropriate immunogenic sites by antibody binding. The reassortment that occurs in hosts with multiple concurrent infections can lead to a new subtype of virus or "antigenic shift". In antigenic shift, the influenza virus acquires a new HA or NA protein also allowing evasion of the immune system through new immunogenic sites that are no longer recognized by the host antibodies[9].

Influenza and the immune system of the lung

The respiratory tract of humans is comprised of two general regions: upper respiratory tract and lower respiratory tract. The upper respiratory tract refers to the nasal cavity, mouth, and pharynx and continues to the tracheal region. The lower respiratory tract is comprised of the trachea to the bronchial tree and lungs [10]. Humans and mice have similar gross anatomy of the lung with five lung lobes. In humans, the right lung is comprised of 3 lobes and receives 55% of the breath, whereas the left is comprised of 2 [11]. Mice have three lobes on the right and one large lobe on the left side with a central lobe between the left and right lobes termed the caudal lobe [12] . Following aspiration,

where liquid is accidentally inhaled, the caudal lobe of the mice will show evidence of the aspiration. The left lobe is anatomically different from the right in both humans and mice to accommodate the location of the heart.

Microscopically, the lung trachea and bronchi are lined by pseudo-stratified ciliated epithelium. Cells present in the airways are columnar and ciliated cells with mucus producing cells (goblet cells) distributed throughout and increasing in number with some types of often chronic infection and irritation of the lung. The trachea, bronchi, and larger bronchioles are lined with ciliated epithelia that utilize a ciliary beat to remove debris, dirt, and pathogens from the lungs. Submucosal glands are present in upper respiratory tract and trachea and bronchi in humans and are responsible for the production of the majority of bronchiole secretions including secretory IgA and are comprised of serous cells. Submucosal glands are not present in the bronchi and lung of mice and other rodents. Clara cells are present in the small bronchioles. These cells are secretory and are able to differentiate into either ciliary cells, which are columnar and have cilia to remove debris and mucus, or goblet cells, which produce mucin, depending on the lung environment (infection, inflammation, etc.). Clara cells secrete proteins (surfactant proteins) that constrict or dilate the bronchiole and other proteins (cytokines, mucins, and anti-microbial peptides) that protect against inflammation and infection [11,13]. Clara cells produce clara cell 10 kD protein (CC10) which is immunomodulatory. Clara cells also have abundant amounts of smooth endoplasmic reticulum that is used for biotransformation of toxins by cytochrome P450 activity. The predominant cell in the lungs of non-primate laboratory animals are Clara cells Mice and humans have differences in the number of Clara cells present in the lung, ~50% and

~22% respectively. [14,15]. The alveolar lumen surface is composed of alveolar type I and alveolar type II cells. Type I cells are very thin and elongate and type II cells are polygonal to cuboidal. All these cells work together to provide an environment in the lung that is conducive to oxygen exchange.

Innate immune cells are also present in the lungs, and include resident alveolar macrophages, dendritic cells (DC), and natural killer (NK) cells. Epithelial and immune cells work in tandem with immune proteins (such as collectins, proteins that opsonize pathogens), surfactant and mucus proteins (MUC5) produced by epithelial cells to provide a pathogen free environment for the lung while allowing for natural host microbiota to coexist. Alveolar macrophages continually sample the lung environment for potential pathogens, removing debris, and clearing apoptotic cells from the lung. Alveolar macrophages are able to sample the environment from the alveolar lining. Resident DCs, DCs that are not migratory and are retained in the lung, sample to the lumen of the airway using dendritic projections between epithelia and migrate to the draining lymph node for antigen presentation. NK cells kill infected cells which are then cleared by alveolar macrophages. Other cell types, such as T cells, B cells, and intravascular macrophages are also present in the lung, although the numbers of these cells present changes dramatically upon infection.

Mucosa-associated lymphoid tissue has two distinctions in the respiratory tract: Nasal associated lymphoid tissue (NALT) and Bronchus-associated lymphoid tissue (BALT). These are lymphoid tissues that are located in the nasal cavity and bronchi, respectively. These areas of lymphoid tissue function similar to lymph nodes; however, they are predominantly unorganized lymphoid tissue marked by T and B cell regions.

Humans and mice do not have BALT in normal conditions; however, BALT can be formed in an inflammatory process and referred to as iBALT or inducible BALT [16,17]. The function of these tissues is to provide a lymphoid follicle of undifferentiated B cells to the site of infection for an expedient response to antigen presented by follicular DCs through the antigen acquisition by M cells [11]. Mice that have formed iBALT have been shown to have a more rapid immune response and recovery from respiratory infections [16]. DCs found in the NALT have been shown to regulate the T Helper 1 (TH1) and T Helper 2 (TH2), T helper cell subsets that produce cytokines that dictate the immune response, in mucosal tissues through notch ligand signaling when exposed to ovalbumin (OVA), a protein found in eggs, given intranasally [18]. These mucosal associated lymphoid tissues provide the immune system with an immune response that is more protective and less pathogenic than a response mounted in lymph nodes [17].

Initial infection with influenza requires the inhalation of virus particles. These particles attach to nasal cavity, trachea/bronchial tree, or can also be swallowed as they enter the host. The virus particles attach via the speculated 2-6αGal/HA interaction. The virus gains entry into the cell and begins taking over the cell machinery to produce large numbers of virions. Budding of the virus from a cell requires the NA protein as well as the apical layer of the host epithelium to form the viral envelop and to allow for dispersion from the cell [4].

Influenza infection invokes a variety of changes in the lung in response to the infection. With influenza infection, extent of immunopathology in the lung is dependent on balance of the immune response and viral clearance. An overzealous immune response characterized by increased cellular infiltration and pro-inflammatory

cytokines/chemokines, molecules that signal to the cells to home to the site of infection, leads to greater immunopathology in the lung, in contrast to a dampened immune response or lack of immune response that leads to a greater viral load and more severe immunopathology in the lung as well.

Because lung tissue is constantly encountering dust and particles with each breath, alveolar macrophages and resident DCs continually monitor and sample the lung environment. Alveolar macrophages and lung epithelial cells are the first responders of the infection of the innate immune response in the lung [10]. Innate immune responses are not specific to a particular pathogen but recognize pathogen associated molecular patterns (PAMPs). PAMPs are proteins and molecules that are conserved and present on pathogens such as lipopolysaccharide in bacterial cell walls and viral RNA in viruses and are recognized by receptors to initiate the immune response. Interferons (IFN) are produced by plasmacytoid dendritic cells (pDC), specifically IFN-α and IFN-β. IFN-α and IFN-β are potent anti-viral proteins. Influenza virions are detected by pattern recognition receptors (PRRs) that recognize PAMPs. These PRRs signal to initiate the innate immune response. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) recognize single stranded ribonucleic acid (ssRNA) and viral proteins, respectively. TLR7 receptors distributed in NK and pDCs recognize ssRNA intracellularly. Double stranded viral RNA (dsRNA) is recognized by TLR3 receptors and retinoic acid inducible gene I (RIG-I) in infected epithelial cells during viral replication. The nod-like receptor family protein 3 (NLRP3) forms an inflammasome, an intracellular vacuole that is involved in apoptosis and inflammation and has been associated with clearance of influenza virus and production of interleukin-1β (IL-1β) and found in human and mouse

alveolar macrophages and in human airway epithelial cells [19]. Production of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) by DCs and macrophages is essential for mounting the immune response with CD8 cytotoxic T cells for effective viral clearance [20]. Cellular immunity to influenza also plays a role in the defense against influenza infection. Influenza specific T helper cells are pivotal at activating the adaptive humoral response to influenza.

Influenza infections promote a strong Th1 cellular response. Th1 responses are characterized by increases in interferon γ (IFN-γ), interleukin 12 (IL-12) and interleukin 2 (IL-2). Th2 responses are defined by increased production of interleukin 4 (IL-4), interleukin (IL-5), and interleukin 13 (IL-13). Th1 responses enhance the effectiveness of CTL responses by presenting antigen to CTL in the context of MHC class I molecules and has pro-inflammatory characteristics. Th2 driven responses enhance humoral immunity with cross presentation to B-cells and utilizing the MHC class II antigen presentation. Th2 responses have also been associated with allergic responses and immune responses to parasite infections.

The role of pDCs, an innate immune cell that recognizes viruses through TLR7, in resolving influenza infection is currently in dispute. While dogmatically accepted as anti-viral in nature, the ablation of pDCs using monoclonal antibodies specific for pDCs in BALB/c mice provided no evidence of impact on the disease outcome in the short term (study was terminated at day 6) and revealed a decrease in interferon α (IFN-α) production coupled with a decrease in viral lung load compared with normal mice [21]. The immune system often compensates by increasing other cell populations and the detrimental effects of the decrease in pDCs may not have been revealed in the short term.

pDCs also function as a regulatory cell by increasing production of anti-inflammatory cytokines such as IL-10 in the later phases of infection. It remains unknown if these cells are recruited or differentiate from resident DCs or are monocyte derived. Monocyte derived dendritic cells (CCR2+ DCs) are recruited to the lung during influenza infection. CCR2 (also known as monocyte chemotactic protein -1 or MCP-1) receptor positive cells have been associated with increased immunopathology in influenza infections demonstrated by decreased lung injury in CCR2 deficient mice [22]. A delicate balance is necessary to recruit and activate dendritic cells in response to infection without the deleterious effects of over recruitment.

Natural killer cells (NK) and CD8 cytotoxic T cells play a role in viral clearance and possibly in the severity and immunopathology of influenza infection. NK cells have been shown to be pivotal in viral clearance and serve to recruit CD8 cytotoxic T-cells to the site of infection and signal to lung dendritic cells [23,24]. Interleukin 22 (IL-22) production by NK cells has been shown to be reduced during influenza infection in the lung; while the mechanism is currently unknown, it is thought to decrease the number of host cells available for infection by the virus [25]. Without NK cells, infections with influenza are markedly more severe. A recent study shows that an increase in NK due to higher viral load in the lung results in increased severity of lung damage [26]. These studies highlight the importance of balance in the NK, NKT and CD8 cytotoxic T cell populations during influenza infections.

Influenza has been shown to infect a variety of host immune cells including macrophages, DCs, and NKs [27-29]. By infecting the immune cells, influenza drives a change in the expression of cytokines and chemokines by the infected cell and may evade

immune detection. NK cells infected by influenza demonstrate a decrease in IFN-γ production [27]. This can lead to increased severity of influenza infection as increases in IFN-γ production during the early onset of infection have been found to be advantageous [30].

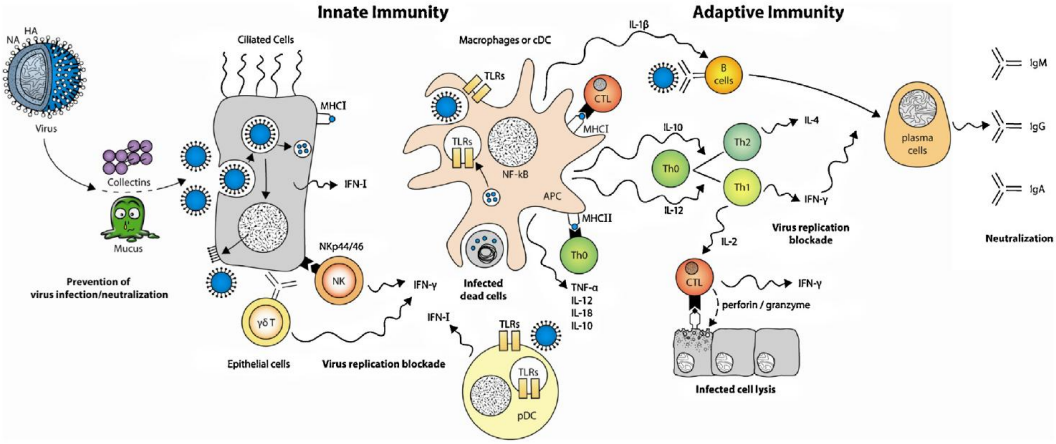
Immunopathology of influenza virus infections has been associated with an unregulated host immune response more so than the infection alone as demonstrated by recent studies. One such mechanism has been referred to as the "cytokine storm". This cytokine storm is the over production of inflammatory cytokines and chemokines by the host immune system due to the infection with influenza. When left unregulated this "cytokine storm" accentuates the inflammation, recruits inflammatory cellular infiltrate and essentially obscures the small bronchioles in the lung leading to cellular necrosis and edema and acute lung injury. Current theory on the cytokine storm has been focused on the innate cellular response to influenza. Recent studies have demonstrated that the cytokine production is originated by the pulmonary endothelium in absence of the cellular response [31]. Other studies focus on the T cell response to the infection as modulators of the cytokine storm through hyperactivity [32]. TLR4 mediated immune response through the adaptor molecule TRIF (TIR domain containing adapter-inducing interferon β) have also been a potential source of acute lung injury in influenza infection by signaling through oxidized phospholipids resulting in the activation of nuclear factor kappa-B (NFκ-B), a transcription factor, and production of IL-6 [33,34]. Pathogenic and pandemic strains have often been linked with a characteristic cytokinemia, increased release of pro-inflammatory cytokines such as IFN-γ, IL-6, TNF-α. These

overabundances of cytokines and chemokines result in an overactive immune response, inflammation, and cellular recruitment leading to acute lung injury.

Secondary bacterial infections with *Streptococcus pneumonia*, *Streptococcus aureus*, and *Hemophilus influenza* have also been associated with increased morbidity and mortality with both seasonal and pandemic influenza infections [35,36]. Acute lung injury caused by the influenza viral infection can allow for opportunistic bacteria to invade the damaged tissue. Superinfection with bacteria has been a hallmark of lethal influenza infections [35]. Increased production of macrophage inflammatory protein 2 (MIP-2 or CXCL2) has been associated with increased susceptibility to secondary bacterial infections. Although the mechanism for the bacterial susceptibility is not known, a study using C57BL/6 mice model of co-infection with *Bordetella parapertussis* and the influenza A (A/FM/1/47) blockage of MIP-2 with anti-mouse antibodies demonstrated a decreased pulmonary infiltration of neutrophils, pulmonary inflammation and decreased illness severity measures in mice treated with anti-MIP-2 compared to mice who were co-infected without the anti-MIP-2 [37].

Severe cases of influenza have resulted in a diffuse alveolar damage (DAD). Mutations in the virus H1N1 associated with 2009 pandemic were demonstrated to infect alveolar epithelial cells and cause DAD. Several factors were linked to an increase in lung lesion presence and severity including increased signaling through TLRs, NFκ-B, prolactin, IFN, Janus kinase/signal transducers and activators of transcription (JAK/STAT), complement, granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 9 (IL-9) and interleukin 17 (IL-17) [38]. Increase in lesions with pandemic strains adds to the morbidity and mortality associated with these viruses. DAD

and acute lung injury (ALI) following influenza infection lead to acute respiratory distress syndrome (ARDS). ARDS has been identified as a complication of severe influenza infection and pneumonia and is linked with variant strains that are able to infect alveolar epithelial cells with production of large amounts of IFN-γ, TNF-α and IL-6 during the late stages of infection [39].



The graphical representation shown above of influenza infection is from Crisci et al, 2013 Molecular Immunology [40].

Influenza epidemiology and treatment

The pandemic influenza outbreak of 2009 reminded the global population about the importance of having reliable supplements and medications to prevent and treat the symptoms of this potentially deadly virus. The CDC reported that 2012-2013 influenza season might have been the worst seasonal epidemic outbreak in the last decade based on the numbers of influenza cases recorded and hospitalization data which reiterated the importance of treatments and preventatives to the public. Current approved preventatives are limited to seasonal vaccination, requiring individuals to be re-vaccinated every year

with a vaccine designed to predict the strains of influenza that may be circulating through the population during the influenza season that typically lasts from November to April. Shortages of anti-viral remedies, such as Tamiflu®, demonstrate the need for further treatments for influenza.

Clinical symptoms of influenza infection include headache, muscle aches, high fever, and extreme fatigue. These symptoms are fairly common among viral infections and leads to the theory that influenza infections are highly unreported. Anyone is susceptible to infection with the seasonal influenza, although the pediatric, geriatric, and immune compromised populations are at the highest risk for complications and possible fatality from this virus. This is dramatically different from the pandemic influenza strains that typically impact the teenaged to middle aged populations.

While progress on a universal vaccine is ongoing, current preventatives for influenza infection require seasonal vaccination with vaccines that consist of the virus strains that are predicted to emerge in during the influenza season. Several antiviral medications exist; however, some viral strains have become resistant to these treatments. These treatments target the basic structures of the virus including the NA and M2 ion channel structures. Treatments targeting the ion channels have been discontinued due to the developed resistance. These drugs include amantadine and rimantadine. While some influenza A and B viruses have started demonstrate resistance against the neuraminidase inhibitors, the majority of these are still susceptible if the treatment is started early during infection. These treatments include zanamivir and oseltamivir. Other drugs that target virus replication and transmission are being investigated [41]. Due to the high mutation

rates of influenza and the potential for viral resistance to current therapies, the need for alternative medicines and preventatives remains high.

Other alternative preventative strategies/natural treatments have been utilized over the years from garlic, vitamin C, vitamin D, exercise, to anti-inflammatories with varying success. In MDCK cultures, garlic extracts have been shown to decrease influenza A/New Caledonia/20/99 (H1N1) virus growth at 10 μg/ml concentration when applied directly to the virus [42]. Vitamin C has been used in studies with influenza. One study utilized a genetically modified mouse strain on a C57BL/6 background to assess the role of vitamin C in influenza infection. The mice were genetically deficient in Vitamin C (with and without administration of Vitamin C) and infected with H3N2 A/Hong Kong/1/68. Increased viral titers were observed in mice deficient in Vitamin C (without administration of Vitamin C) and had lower IFNα/β levels in the lung than mice who received Vitamin C [43]. Vitamin D has been evaluated as a supplement to vaccination strategies to enhance the efficacy of vaccination, as well as, a supplement to restore vitamin D levels in hypovitaminosis in obesity [44].

Exercise has also been studied in the context of influenza infection prevention and vaccination enhancement. The level of exercise becomes an important factor in addressing the effect of exercise on infection and vaccination enhancement, similar to affect the dosage of a vitamin or medication would have on the outcome. Moderate exercise has been shown to enhance the antibody levels in older adults after vaccination with influenza vaccine [45]. In treadmill exercised mice, a reduction in mortality, cytokines and influenza specific antibody was observed after infection with influenza A/PR/8/34 virus. Despite the reduction in antibody, these mice were protected on

secondary challenge with the same virus[46]. While moderate exercise can be protective and beneficial to influenza infection, exhaustive exercise has been shown to be deleterious to the outcome of mice infected with influenza resulting in a higher mortality rate [47]. This reiterates the importance of establishing a dose not only for oral supplements, but also for exercise.

Another common treatment used in influenza infection is the use of anti-pyretics or non-steroidal anti-inflammatory (NSAID) medications, such as ibuprofen and acetaminophen, to reduce the clinical symptoms associated with influenza infection. There is debate on whether ibuprofen is beneficial or harmful in the context of influenza infection [48]. It may prolong the infection due to the reduction in fever and the optimal temperature of influenza replication. A study with paracetamol, or acetaminophen that inhibits COX-2 and prostaglandin formation, was conducted using C57BL/6 mouse strain model of influenza infection. In this study, mice treated with paracetamol demonstrated decreased influenza related inflammation and cellular recruitment in the lung compared to mice treated with celecoxib, a selective COX-2 inhibitor, and mice without treatment. These mice also had protective immunity to secondary challenge with the same virus [49]. Other anti-inflammatory treatments could provide similar benefits.

Echinacea history and usage

Interest in natural remedies for ailments and diseases such as respiratory illnesses has been on the rise in the last decade. Treatments such as *Hypericum*, *Prunella*, and *Echinacea* have gained popularity. Studies have tried to confirm the efficacy of these treatments, but have produced widely variable results.

Echinacea species are a group of herbaceous plants in the family *Asteraceae*. Native to the United States, *Echinacea* species grow commonly in the wild and have been cultivated for medicinal use for centuries. The Native Americans were the first to use the plant to treat respiratory ailments such as colds or flu by boiling in water or directly consuming the roots of the plant. Used topically, it has also been used to treat skin ailments, wounds, and burns [2].

Variations of effectiveness reported in the literature could be attributed to the variety of species, plant parts, and preparation techniques available. There are nine known species of *Echinacea*. Three of these have shown to be of medical importance: *E. purpurea*, *E. angustifolia*, and *E. pallida* commonly known as the purple coneflower, the narrow leaf coneflower, and the pale purple coneflower, respectively. Various parts of the plant can be utilized for each preparation: the whole plant, stem, roots, leaves, and aerial parts. The part of the plant selected can then be extracted by boiling in water or through an ethanolic extraction preparation. Each of these preparation techniques creates extracts with different constituents present. Water preparations yield extracts with hydrophilic compounds such as polysaccharides. Ethanolic preparations yield extracts with lipophilic compounds such as alkamides, caffeic acid derivatives, and other phenolic compounds. Both water and ethanolic preparations contain each of these constituents. The extraction method dictates which compounds will predominate in each mixture. Even growing season has been demonstrated to impact the nutrients and compounds present in an extract [2,50,51]. Longer growing seasons have a reduction in alkamides and increase in phenolic compounds, whereas detrimental growing conditions, such as drought, have been shown to increase chicoric acid content of *E. purpurea* roots[52].

With all these variables to consider, evaluating the efficacy of an *Echinacea* extract can pose a challenge.

Echinacea extracts and immunity

Many studies with *Echinacea* species have shown the enhancement of cellular innate immunity. Enhanced phagocytosis has been demonstrated *in vivo* and *in vitro* with the application of *Echinacea* extracts. Adaptive immunity has largely been unexplored; however, some studies have shown that T cell maturation, B cell proliferation and antibody production remain unchanged when treated with *Echinacea* [53] Variations of efficacy in the literature can be explained through the varied species, plant anatomical parts, and extraction methods used for each study. These studies are further complicated by variations in animal models utilized and immune parameters measured. Active chemical components have been isolated from *Echinacea* and tested for immune modulation. While the mechanisms of the whole extract remain to be elucidated, several chemical constituents have been researched for immune stimulatory (polysaccharides) and anti-inflammatory properties (alkamides and caffeic acid derivatives). Other properties such as phagocytosis, cytokine/chemokine expression, cellular migration, and cellular proliferation and differentiation have also been explored with polysaccharides, alkamides, caffeic acid derivatives, ketones, and phenolics.

Hydrophilic compounds in Echinacea extracts and the immune response

Echinacea water extracts are rich in polysaccharides. Although each species has varying chemical constituents and chemical concentrations, several compounds remain consistent among the genus. Polysaccharides isolated from *Echinacea* species have been

shown to enhance the innate immune response through activation and enhanced activities of phagocytes, but have not been shown to enhance adaptive cellular or humoral immunity. Polysaccharides (xyloglucanes and one arabinogalactane mixed at the ratio of 2:1) isolated from *E. purpurea* given *in vivo* (by intravenous injection into test subjects) enhanced the proliferation of macrophages in presence of GM-CSF *in vitro* and enhanced the phagocytic activity of the human macrophages *in vivo* and *in vitro* [53]. While these polysaccharides were able to modulate the non-specific immune response, these polysaccharides (xyloglucans and arabinogalactan) were not found to enhance antibody production to sheep red blood cell stimulation in male BALB/c and LPS resistant C3H/HeJ mice[54]. Studies of the adaptive cellular and humoral immune responses with *Echinacea* extracts need to be further explored.

Several *Echinacea* polysaccharides have been further characterized and found to have immunomodulatory activities: fucogalactoxyloglucans and arabinogalactan. The immune stimulating properties of these polysaccharides have been explored in the non-specific innate immune cells such as macrophages and NK cells. The fucogalactoxyloglucans were found to increase phagocytosis, and arabinogalactan stimulated TNF-α production in macrophages [55,56]. Larch tree derived arabinogalactan was also shown to increase nitric oxide production and enhanced phagocytosis by RAW 264.7 murine macrophages *in vitro* [57]. Arabinogalactan stimulates the production of IL-1β, IFN-β and TNFα in murine macrophages and stimulates NK cells [58]. NK cell production and function was enhanced by the supplementation of *Echinacea* in BALB/cByJ male mice (without exposure to pathogens or immune stimulants), which was considered the attribute that added to the longevity of

the mice receiving the *Echinacea* from youth [59]. Similarly, aged DBA/2 male mice fed ground *E. purpurea* for 14 days were assessed to have increased number of NK cells in the bone marrow and spleen with increased cytotoxicity to labeled tumor cells compared to mice receiving control [60]. Additional studies have shown that the polysaccharide arabinogalactan activates complement in an *in vitro* complement activation assay [61].

While the signaling mechanisms of *Echinacea* polysaccharides in macrophage activation remains unclear, several mechanisms have been suggested for other plant derived polysaccharides. Signaling through pattern recognition receptors such as mannose receptors, complement receptors, scavenger receptors, Dectin-1, and TLR4 have all been suggested mechanisms [62]. These receptors signal downstream resulting in activation of pro-inflammatory cytokines, such as IL-1β, IFN-β and TNF-α, NFκ-B, and enhancement of phagocytosis.

Other cellular immune responses have been evaluated for enhancement by *Echinacea* water preparations. More recently, dendritic cells (DCs) have become a focus for immunostimulatory effects of *Echinacea*. Polysaccharides in *Echinacea purpurea* extract (150 μg/ml and 450 μg/ml of the root extract) applied in culture may be responsible for the activation and maturation of dendritic cells in C57BL/6 and OT II/Thy1.1 male mice challenged with OVA evidenced by the increase in stimulatory markers such as CD86, MHC II and CD11c in the *E. purpurea* treatment group compared to vehicle [63].

Echinacea enhances cellular activation and also modulates the production of cytokines and chemokines. Extracts are able to both enhance the expression of

cytokines/chemokines as well as suppress pro-inflammatory cytokines in response to infections owing to the different immunomodulatory effects observed with the extracts. Water extracts from *Echinacea purpurea* were able to stimulate the production of IL-6, TNF-α, IL-10, and MIP-1α from BALB/c mouse non-adherent splenocyte cultures treated for 48 hours with 1 mg/ml *Echinacea* root and leaf extract without exposure to stimuli or pathogens [64]. Echinaforce®, a commercially available *Echinacea* water preparation, showed anti-bacterial activity against respiratory pathogens S*treptococcus pyogenes*, *Hemophilus influenzae*, and *Legionella pneumophila* and demonstrated anti-inflammatory properties with decreased bacterial infection associated inflammatory cytokines IL-6 and IL-8 *in vitro* [65]. Increased total white blood cell counts were observed in Sprague–Dawley rats fed *Echinacea* aerial parts (50mg/kg) of a commercial preparation and proprietary blend (Nature's Resource) over the course of an eight week study with lymphocytes and monocytes populations increasing in number; while also observing an increased serum IL-2 level compared with control rats [66]. The polysaccharides present in the extracts have been demonstrated to have immune stimulating properties with the increase in white blood cell counts, increase in lymphocyte and monocyte populations, and pro-inflammatory cytokines/chemokines.

Specific polysaccharides derived from *Echinacea* have not been evaluated in the context of adaptive humoral immunity. However, arabinogalactan isolated from other plant species has been evaluated as an adjuvant to vaccination with pneumococcal vaccines. In a human trial of a proprietary arabinogalactan extract (isolated from Larch tree), increased levels of antibody (IgG) to *Streptococcus pneumonia* were seen in the group receiving the arabinogalactan compared to control without evidence of increased

serum cytokines, white blood cells, or salivary IgA [67]. This observation could give credence to the potential of arabinogalactan from *Echinacea* having an immunostimulatory effect on the adaptive humoral immunity as well.

Caffeic acid derivatives in Echinacea extracts and immune function

Caffeic acid derivatives are another important class of constituents commonly found in *Echinacea* extracts. Caftaric acid, chlorogenic acid, chicoric acid, cynarin and echinacoside are all caffeic acid derivatives found in *Echinacea* species. The concentrations of each of these derivatives vary from species to species. In general, *E. angustifolia* and *E. paradaoxa* contain chlorogenic acid and echinacoside, and lack chicoric acid and caftaric acid. *E. angustifolia* extracts have been shown to contain cynarin. *E. purpurea* extracts contain caftaric, chlorogenic, and chicoric acid, but cynarin and echinacoside have been shown to be absent in extracts prepared from *E. purpurea* [2,50,68]. These molecules are somewhat water soluble phenolic compounds that are ubiquitous in plants and are known anti-oxidants. Caffeic acid derivatives have been evaluated for their effect on innate immune cell populations (macrophages, NKs, and DCs), T cell populations, cytokine/chemokine expression, antioxidant properties, and anti-viral capabilities.

Caffeic acid derivatives have antioxidant properties and modulate cellular growth. Chicoric acid was demonstrated to be an antioxidant scavenges free radicals [69]. Chicoric acid was not able to inhibit pro-inflammatory cytokines to the same extent as alkamides isolated from three different *Echinacea* species: *E. purpurea, E. angustifolia, and E. pallida* due to the decreased inhibition of iNOS and COX-2 by chicoric acid [70].

Caffeic acid (10, 25, 50, 100 μM) and chlorogenic acid (10, 25, 50, 100 μM) were demonstrated to inhibit COX-2 and Fyn kinase, a kinase involved in cellular growth often associated with unregulated cell growth and tumor formation, during UVB exposure in the JB6P+ cell line (mouse skin epidermal cells) with decreased PGE2 concentrations and NFκ-B transcription in a dose dependent manner [71]. Fyn kinase has also been shown to be utilized during differentiation of T-cells; however, the Fyn kinase in T-cells has been characterized as a different isoform [72].

Modulation of T cells by caffeic acid derivatives has been evaluated *in vitro*. In one study, IL-2 production by Jurkat E6.1 cells T cell cultures was evaluated in the presence of caffeic acid derivatives (caftaric acid, cichoric acid, and chlorogenic acid at a concentration of 6.25 to 25μg/mL) with mitogenic stimulation using phytohemagglutinin and phorbol myristate acetate (PHA/PMA). No effect on IL-2 levels was observed with the caffeic acid derivatives; however, chicoric acid was shown to increase proliferation of the Jurkat T cells [73]. Increased NFκ-B expression was observed using Jurkat T cell cultures in response to stimulation with LPS or PMA and treatment with chicoric acid (0.8 or 8 μg/ml) [74]. Some caution should be used in interpreting this data since chicoric acid has poor bioavailability. NFκ-B positively regulates the expression of cytokines and chemokines by the T cells and also the expression of adhesion molecules. Taken together chicoric acid may enhance the proliferation and differentiation of T cells.

The role of caffeic acid derivatives on dendritic cells has been evaluated *in vitro*. Isolated human dendritic cells from patients sensitized to dust mites were exposed *in vitro* to LPS or dust mite challenge and treated with caffeic acid derivative from

Echinacea purpurea (10 μM -100 μM) in dimethyl sulfoxide (DMSO). The caffeic acid was shown to decrease in a dose dependent manner the production of IL-12 and IL-10 in lipopolysaccharide (LPS) challenged monocyte derived DCs compared to IL-10 and IP-10 in the dust mite challenged DCs through decreased signaling in the NFκ-B pathway [75]. A butanol fraction of *Echinacea* extracts were used to assess the activation of iDCs through increased expression of the maturation marker CD83. Caffeic acid, chlorogenic acid and chicoric acid were present in this butanol fraction (1, 10 or 50 μg/ml) and may play a role in the increased activation of iDCs in *Echinacea purpurea* extract application on human DCs *in vitro* [76]. Although further investigation is necessary, the enhancement of dendritic cells by *Echinacea* may enhance the adaptive immune response to infections.

Anti-viral activity of *Echinacea* extracts has been demonstrated *in vitro* but does not translate to *in vivo* models. Chicoric acid may be involved in the anti-viral activity seen *in vitro*. Although cellular uptake of cichoric acid is inefficient and slow, chicoric acid has been shown to be anti-viral *in vitro* at 1 μM against human immunodeficiency virus 1 (HIV 1) by inhibiting integrase, an enzyme required for viral replication, and thus, preventing viral integration *in vivo* [77]. Polysaccharides in *Echinacea* also demonstrate anti-viral activity. Polysaccharides from *Echinacea* have been thought to influence the macrophage function and contribute to the direct anti-viral activity [78]. Fractions containing polysaccharides and chicoric acid from *Echinacea* extracts have been shown to have anti-viral activity against influenza and herpes simplex virus-1 [79]. Alkamides and ethanol fractions of *Echinacea* have been shown to have anti-viral activity against influenza, but not as robust as the chicoric acid and polysaccharide fractions [78]

Lipophilic compounds in Echinacea extracts and Immune Response

The lipophilic compounds alkylamides (isobutylamides) and polyacetylenes are found in the ethanol preparations of *Echinacea* extracts. These compounds have been associated with the anti-inflammatory properties of *Echinacea* extracts. Debate has ensued over the efficacy of ethanoalic extracts and whether the ethanol has the anti-inflammatory effect. This criticism has been addressed by utilizing ethanolic controls containing the same concentration of ethanol that was used with the extracts.

Anti-inflammatory effects due to alkylamides and polyacetylenes have been evaluated in macrophages, T-cells, and cytokines/chemokines. Alkamides (isobutylamides) isolated from *Echinacea purpurea* were able to inhibit the production of inflammatory cytokines from RAW 264.7 murine macrophages *in vitro* [80]. *Echinacea* polyacetylenes (ketones), particularly ketone 23, isolated from *E. angustifolia* and *E. pallida* extracts, were able to inhibit PGE2 production in RAW264.7 with LPS challenge [81]. In a study using RAW 264.7 and influenza A/PR/8/34 infections, alkylamides decreased the production of TNF-α in a dose dependent manner [80]. IL-2 production by Jurkat E6.1 cells T-cell cultures was evaluated in the presence of ethanolic *E. purpurea* extract and several alkamides with mitogenic stimulation using PHA/PMA. Decreased IL-2 production was observed with the whole *E. purpurea* extract and alkamides (isobutylamides) [73]. Pro-inflammatory cytokines have been shown to be inhibited by whole ethanolic extracts. Ethanol based extracts from *Echinacea purpurea* inhibited IL-6 production in BALB/c mouse splenocyte cultures [64]. The decrease in IL-2 and inflammatory cytokines from macrophages together may depress the immune response.

Aerial parts of *E. purpurea* were extracted in a water/70% ethanol combination and reconstituted in phosphate buffered saline (PBS) to be given to Female C57BL/6 mice infected with influenza A/WSN/33 (H1N1) for 5 days post infection beginning on the day of infection. Mice receiving the extract demonstrated a decreased amount of IFN-γ, IL-10, and KC both in the lungs and serum of infected mice compared to the vehicle control who received PBS only [82]. While these mice did not show decreased viral titer, these mice had reduced weight loss at the late phase of infection. These findings are consistent with results obtained from our laboratory. The capability to decrease inflammatory cytokines/chemokines at the site of infection could be utilized to decrease the inflammatory response at the late phases of infection to reduce tissue damage and recovery time from infections.

Alkamides (isobutylamides and methylbutylamide) inhibit the production of 5-lipoxygenase and cyclooxygenase [83,84]. These enzymes are crucial to the production of prostaglandin (PGE2), and the inhibition of the enzymes results in decreased PGE2. PGE2 is involved in the pain signaling pathway and has been linked with the production of TH2 cytokines [85]. Using RAW264.7 macrophages, alkylamides from *E. angustifolia* were analyzed for production of PGE2 and NO. Bauer alkylamide 11 was shown to decrease the production of PGE and NO from murine macrophages [81]. PGE2 is also a known inhibitor of NK cells. By inhibiting PGE2, NK cells increase in number and function [60]. Increasing NK number and function would stimulate the non-specific immune response and enhance the killing of virally infected cells. This enhancement is in contrast to the inhibition of macrophage cytokines and T-cell proliferation, but may be advantageous during viral infections that rely on NK cells for viral clearance.

Alveolar macrophage enhancement by alkamides has been reported. While RAW264.7 murine macrophages demonstrated an inhibition and anti-inflammatory response to alkamides, alveolar macrophages are stimulated. Increased phagocytosis and increased expression of TNF-α and NO were observed in alveolar macrophages from Sprague-Dawley rats stimulated with LPS and treated with alkamides (12μg/kg/day for 4 days) isolated from *E. purpurea* [86,87]. When the same alkamides were applied to the splenocytes, no changes were observed. This suggests that the cellular source of macrophages exposed to the extract components are also a factor in the effect the extract has on the immune system. Enhanced alveolar macrophage function may enhance the immune response to respiratory infections.

While the exact mechanisms of immune activation and modulation remain largely unexplored, a recent study utilizing proteomics and genomics evaluated the effect of *Echinacea* extracts on DC trafficking and a stem and leaf preparation of *E. purpurea* in DMSO was demonstrated to enhance the *in vivo* DC trafficking in BALB/c female mice with *in vitro* proteomic analysis suggesting the involvement of JNK2 (c-Jun NH2-terminal kinase 2), PP2C-α (protein phosphatase 2C-alpha), AKT (protein kinase B), ERK (extracellular signal-related kinase) and MAPKAPK (MAPK-activated protein kinase 2) signaling pathways in the increased ability for DC trafficking [88]. These signaling pathways need further exploration for the impact on immune function in the presence of *Echinacea*.

Adaptive immune responses in the context of *Echinacea* treatment have not been evaluated in depth. Primarily, the impact of *Echinacea* on non-specific immune responses has been the focus of research. Male BALB/c mice were gavaged with one of

three *Echinacea* extracts (*E. purpurea, E. angustifolia*, and *E. pallida*) and challenged with sheep red blood cells (sRBC) and immune parameters were evaluated *in vitro* through stimulation with LPS or concanavalin A (Con A). All three extracts demonstrated increased lymphoctyes in spleens, NK cytotoxicity, and antibody responses to sRBC, while *E. angustifolia* and *E. pallida* showed more anti-inflammatory potential with decreased TNF-α and IL-1β and increased IL-10 and Th2 cytokines [89].

Besides direct immune enhancement and modulation, *Echinacea* extracts have been shown to enhance wound healing. Particularly, an alcohol extract of *E. pallida* was given to male SKH-1 mice for three days prior to cutaneous injury and for four days post injury, and then evaluated for stress related wound healing. These mice demonstrated a reduced healing time compared to the vehicle treated group [90]. This wound healing could potentially be beneficial in the course of infection, although further investigation of this possibility is necessary.

Echinacea extracts may be comprised of compounds that have opposing effects depending on the concentration and chemical composition in each extract. The mechanisms for the interaction of these molecules is unexplored and needs further investigation but may contribute to the variability in the results seen between extracts and even intra-extract variability. Despite the variability of preparation and models utilized *Echinacea* demonstrates the potential to be a powerful immunomodulatory supplement when taken in the water phase and anti-inflammatory when taken in the alcohol or tincture phase.

Echinacea compounds bioavailability

Efficacy of *Echinacea* extracts hinges on a variety of variables. Chemical constituents vary by extract preparation, species, and plant parts utilized. Also, bioavailability of the extracts at the site of interest is also a potential obstacle to the efficacy of the extracts. Not all chemical constituents are absorbed and there appears to be molecular interactions that may accelerate or inhibit the active constituents in the extract. Polysaccharides are water soluble and are actively absorbed and digested. Chlorogenic acid present in coffee has been demonstrated to be partially absorbed intestinally while the majority of the molecule is converted to caffeic acid that is readily absorbed or converted to other molecules in the lower gut [71,91]. This is in contrast to the investigation demonstrating the inability of caffeic acid derivatives (chicoric acid, caftaric acid, and echinacoside) to diffuse through Caco-2 monolayers, a cell line utilized to examine the ability of compounds to cross the intestinal barrier, and lack of caffeic acid derivatives found in human serum twelve hours post consumption of *Echinacea* [92]. In H9 or MT-2 cells (T-cell lines used in HIV-1 research), caffeic acid was taken up by the cell lines but with poor kinetics increasing with larger quantities of caffeic acid present [77]. Caffeic acid derivatives from *Echinacea* plants may not be bioavailable when ingested orally and may be more efficacious when delivered directly to the cells of interest. Alkamides are readily absorbed through the intestine and are often used in the dosing of *Echinacea* extracts. *Echinacea angustifolia* alkamides were analyzed using fasting human subjects and were found to be actively and quickly absorbed [93]. Because *Echinacea* is administered orally, the bioavailability of the constituents impacts

the efficacy of the extract. Compounds not readily absorbed by the intestine would not be bioavailable in the lung for effective treatment of influenza infection.

Echinacea dosing

Echinacea dosing remains in debate. In general, the suggestion is to consume the supplement for short durations 1-2 weeks [2]. Although the concern of continual stimulation of the immune system exists, a study using BALB/cByJ male mice explored the effect of long term (life time of the mouse) daily use and concluded that there were no deleterious effects observed with a consistent increase in total NK cells during the duration [59]. For respiratory infection and prevention, the recommended dosing is either a short term prophylactic (dosing before travel or exposure) or an early infection immunostimulant (dosing with early symptoms for best results).

Echinacea side effects and drug interactions

Botanical remedies are often used without regard to side effects or potential drug interactions. Side effects of most herbal remedies are limited and therefore make herbal remedies more attractive to potential consumers. *Echinacea* is similar to other herbal remedies in this regard with few side effects reported. The most common side effect of *Echinacea* is allergic reaction to the extract or preparation [2]. Because of the unregulated nature of herbal remedies, content and constituents present in the preparations available are not standardized or even fully disclosed due to the proprietary nature of many herbal combinations. This adds to difficulty in evaluating the efficacy of preparations available to the public for consumption.

Ultimately, botanical products are taken without regard to interaction with prescribed pharmaceuticals or even other over the counter medications. These interactions can inhibit the effect of prescribed medications or even enhance the effects of other medications. *Echinacea* has been demonstrated to have interactions with pharmaceuticals, particularly those functioning through the cytochrome P450 pathways. Alkylamides (*N*-isobutyldodeca-2*E, 4E, 8Z*, 10*Z*-tetraenamide, Alkylamide 8, isobutyldone) derived from *Echinacea* have been sown to inhibit cytochrome P450 in a concentration dependent manner [94-96]. Pharmaceuticals utilizing the cytochrome P450 include immunosuppressants, anticoagulants, calcium channel blockers (anti-epileptics, cardiomyopathy treatments, blood pressure medications), and benzodiazepines (psychiatric medication given for anxiety, depression, and muscle relaxation). A decreased clearance of caffeine has been shown in conjunction with the use of *Echinacea* extracts [2]. There are some conflicting data in the literature for the interaction of particular constituents of *Echinacea* extracts with cytochrome P450 [94,95,97]. While these interactions are not contraindicated for the general use of *Echinacea* products, the interactions should be noted, particularly in an aging population and the increased use of pharmaceuticals such as calcium channel blockers.

Obesity and immunity

The impact of nutrition on immune function is becoming increasingly apparent. From calorie restriction to diet induced obesity, the calories consumed directly impact the ability of the immune system to fight infections. In a country where obesity is becoming more prevalent, the importance of evaluating the effects of obesity is increasingly important. The CDC estimates that 30% of the American population was overweight or

obese as defined by body mass index (BMI) in 2009-2010 with the obesity trend on the rise [3]. BMI is an indicator based on height and weight that is used to predict risk for obesity, cardiac risk, and other diseases. While in general the BMI is a good predictor of risk, the calculation does not account for body structure and can be misleading in individuals with a smaller or larger bone and body structure. A BMI measurement can be categorized by underweight (BMI <18.5), normal healthy weight (18.5<BMI <24.9), overweight (25<BMI<29.9), and obese (BMI >30) [98].

Several mouse models have been developed to study the role of obesity in immune dysfunction. Genetically altered models (ob/ob and lp/lp knockouts) offer models without leptin signaling. These models have genetic knockouts or mutations of the leptin gene and therefore may not be the most accurate model for translational studies since leptin deficiency is not a common cause of obesity in humans. Diet induced obesity models utilizing high fat diets proffer a more realistic modeling of immune dysfunction in human obesity with increased leptin production seen in the mice. Leptin functions in immune signaling during infection [99]. In diet induced obesity, the increased leptin production leads to a leptin resistance state and impairs immune function.

Obesity is associated with both insulin and leptin resistance states. In obese individuals, increased insulin is produced and cells become resistant to the insulin present in circulation. This requires increased insulin levels for a similar glucose uptake [100]. Similar responses have been observed with leptin. Leptin is critical for the engagement of the immune response and utilizes a pathway similar interleukin 6 (IL-6) to initiate immune responses in the lung [99,101-103]. In obesity, baseline levels of leptin are chronically increased. During infection, the leptin level is not altered in obese C57BL/6J

mice, however, signaling downstream of leptin is attenuated [102]. To investigate the time frame required to induce leptin sensitivity, a diet induced mouse model of C57BL/6 mice receiving a high fat diet for 20 weeks were evaluated at different weeks to determine leptin sensitivity. Mice received intraperitoneal injections of leptin at 1 week and at 8 weeks after initiation of the high fat diet. At 1 week, the mice were sensitive to 2 μg/g mouse weight of leptin. At week 8, the mice were insensitive to the same dose of leptin [104]. It is possible that this downstream signaling could result in a decreased immune response.

Innate cellular immunity is impacted by the obese condition. The ability of NK cells from lean (non-obese) volunteers to kill a tumor cell line (K562) *in vitro* was inhibited in the presence of dose 10 μg/ml leptin [105]. Obese human populations demonstrate a decrease in CD3+, CD8, CD4, and NK cells [102,106]. A similar trend was seen in an obese rat study using hepatitis B surface antigen (HBsAg) vaccination that determined that genetically obese male rats (WNIN/Ob) have decreased cytotoxic CD8+ populations and genetically obese female rats have decreased CD3+ and CD4+ T cell populations in the spleen after challenge with HBsAg *in vitro* following vaccination *in vivo* [107].

The importance of nutrition in immunology is constantly being reiterated. Calorie restriction and obesity both impact the immune system in a negative way. Calorie restriction in mice by gradual underfeeding (up to 40% decrease in caloric intake) has been shown to decrease the ability of the immune response to control viral replication through decreased NK function [102]. This may be a key to increased viral infections (in particular influenza) with ageing populations [108]. NK cell number and function were

also shown to be decreased in obese volunteers compared to lean control group in cytotoxicity studies against a tumor cell line (K562) *in vitro* [105].

Obesity has been associated with an overall inflammatory state. Increases in IL-6 and TNF-α are hallmarks of inflammation and have been demonstrated to be increased in obese individuals in absence of infection. A recent study has shown an increase in suppressor of cytokine signaling 3 (SOCS3) transcription in obese individuals [109]. SOCS3 is involved in leptin signaling and in viral infection. Using peripheral blood white blood cells, Teran-Cabanillas et al stimulated TLR3 and TLR7 using TLR antagonists and found that obese individuals had increased SOCS3 and decreased production of IFN-α/β [109]. These findings suggest that obese individuals would produce less IFN-α/β in response to influenza infections leading to a higher viral load and consequently increased morbidity in obese populations. Despite similar viral titers, diet induced (high fat/high sucrose for 22 weeks prior to infection) obese C57BL/6J mice infected with influenza A/PR/8/34 showed a decrease in IFNα/β, MCP-1, RANTES, and NK cell function in the lungs at day 3 post-infection compared with C57BL/6J mice who received low fat/low sucrose diets [110].

Memory CD8 T cells are important in protecting a host from a secondary infection. Two types of memory T cells have been categorized: effecter memory and central memory. Reductions in the effecter memory cell populations could lead to impaired secondary immune responses. In diet induced obese male C57BL/6J (received high fat/high sucrose diet for 20 weeks prior to infection), number of effecter CD8 memory cells decreased faster post infection in obese mice compared to lean control mice despite elevated levels of IL-15 and similar levels of central memory T cells [111].

Male, db/db, diabetic mice, were used to evaluate the impact of leptin resistance in the formation of acute respiratory distress syndrome (ARDS) following lung infection. These mice have no leptin receptor and therefore have increased circulating leptin concentrations [99,103]. This model has been used as a leptin resistance model. Diabetic mice and humans have demonstrated a decrease in ARDS, and leptin resistance has been implicated as the precipitating factor by inhibiting TGF-β initiated fibroproliferation and enhancing the effects of peroxisome proliferator activated receptor gamma, PPAR-γ [112]. Leptin resistance is a consequence of long term diet induced obesity. The duration of the diet to acquire leptin resistance remains under investigation.

Overall, obesity immune dysfunction impacts both the innate and adaptive immune responses. These dysfunctions either individually or in complex could lead to the increased morbidity and mortality associated with influenza infection in obese individuals. It is unknown whether resolving just one of the dysfunctions (the innate or adaptive dysfunction) would decrease disease severity and lead to influenza resolution.

In this dissertation, the effects of *Echinacea* treatment on influenza infection *in vitro* and *in vitro* were evaluated utilizing several different species of *Echinacea* using both water and ethanol preparations of each species. Several *in vitro* methods were utilized to determine the effect of *Echinacea* directly on lung epithelial cells and mix cellular cultures. A combination of water and ethanol extracts was administered to mice infected with influenza virus to assess the efficacy of modulating the immune response during different phases of infection (early vs. late). Lastly, *Echinacea* extracts were evaluated for efficacy in diet induced obese mice to determine if *Echinacea* could correct obesity associated immune impairment. With respect to the in vitro studies, it was

hypothesized that *Echinacea* would exhibit antiviral activity, that the water extracts would increase chemokine production by influenza-infected lung epithelial cells, and that the ethanol extracts would modulate spleen responses to induced a decreased in inflammatory cytokine and chemokines. It was hypothesized that *Echinacea* water extracts would stimulate the immune response to influenza infection and the ethanol extracts would attenuate the influenza-associated lung inflammation. In the obese model, it was hypothesized that the *Echinacea* extract treatment would enhance the immune response and would correct the obesity associated immune impairments associated with influenza infection in obese individuals.

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CHAPTER II. IMMUNOMODULATORY PROPERTIES OF THREE *ECHINACEA* SPP. DURING INFLUENZA INFECTION *IN VITRO*

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Abstract:

Background: *Echinacea* is a popular dietary supplement typically used to treat respiratory infections. However, the potential immunomodulatory and/or anti-viral properties of *Echinacea* may vary by species and/or type of extract, and are not well-characterized in response to respiratory viral infection. In this investigation, the antiviral and immunomodulatory effects of *Echinacea* in response to Influenza A H1N1 infection were identified for 3 species of *Echinacea* (*E. purpurea, E. angustifolia,* and *E. paradoxa var*. *paradoxa),* comparing water and ethanol preparations for each species. Three potential pathways by which *Echinacea* may protect against Influenza infection were evaluated: 1) direct antiviral action, 2) enhancement of host defense in respiratory epithelial cells, and 3) alteration of innate or adaptive immune response.

Methods: Water and ethanol extracts were prepared from each of the *Echinacea* species. To assess direct antiviral response, Influenza-permissive MDCK cells were incubated with *Echinacea* extracts prior to virus infection with Influenza A/PR/8/34 H1N1 to determine the extent to which plant extracts may inhibit viral growth. A549 respiratory epithelial cells were cultured with *Echinacea* plant extracts plus influenza virus to measure cytokine and chemokine response to infection. An *ex vivo* culture of spleen cells harvested from mice infected with Influenza A/PR/8/34 or from non-infected mice was used to evaluate the effect of *Echinacea* treatment on influenza-induced cytokine production.

Results: The *E. purpurea* H2O extract demonstrated limited anti-viral activity *in vitro* at both low and high concentrations tested, whereas *Echinacea* EtOH extracts from all three species demonstrated anti-viral activity only at high concentrations of extract. The A549 cells responded to influenza infection by increasing the production of IL-6, IL-8, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP1β, RANTES, and VEGF. *Echinacea* water extracts from all species enhanced chemokine production influenza-infected A549 cells (eotaxin, GM-CSF, IL-8, MIP-1α, and MCP-1) to a greater extent than ethanol extracts from the same species. In spleen cells obtained from non-infected mice, only water extracts from *E. purpurea* increased IL-17, IL-5, MIP-1α and MIP-1β, whereas water extracts from all species decreased inflammatory cytokines IL-1β, IL-6 and IFN-γ. Ethanol extracts from all species generally decreased cytokine and chemokine production by spleen cells from uninfected mice. When spleen cells were obtained from influenza-infected mice and re-stimulated in vitro with influenza virus, the ethanol extracts from all

three species inhibited cytokine and chemokine production, and water extracts had minimal effects.

Conclusions: Extracts prepared from all three species (*E. purpurea, E. angustifolia, E. paradoxa var. paradoxa)* had similar effects. Instead, the type of extract preparation was more important in determining the immunomodulatory response. The most consistent effects of water extracts were an enhancement of A549 cell chemokine response to influenza virus, and a reduction of inflammatory cytokine production by spleen cells from infected mice. In contrast, ethanol extracts generally had little effect on A549 cell response, but did result in significant inhibition of cytokine and chemokine response in splenocytes obtained from uninfected and infected mice. Based on these findings, it is possible that *Echinacea* water extracts may stimulate early innate host defense to viral infection by increasing respiratory epithelial cells chemokine response, and *Echinacea* ethanol extracts may inhibit infection-associated inflammatory response by host immune cells.

Introduction:

Influenza continues to be a global health concern with the disease being a major cause of morbidity and mortality in the United States and throughout the world [1]. While no universal influenza vaccine is available, annual vaccination is the primary prevention against infection. However, these vaccines are prepared to prevent seasonal infections and may not be available quickly enough during a pandemic. Also, a significant number of individuals do not receive the vaccine and remain vulnerable to infection [2,3]. Although anti-viral medications are available, resistant influenza strains

have emerged [3-6]. Given these limitations with vaccine and anti-viral treatments, along with a recent interest on "natural remedies" by the consumer, *Echinacea* has emerged as a popular dietary supplement for treating colds and influenza. *Echinacea* efficacy has been questioned largely due to the variation in preparations and species available for use, and a potential role for *Echinacea* in improving resistance to influenza infection has not been well characterized.

Some studies have suggested that certain species of *Echinacea* exhibit direct anti-viral effects on influenza virus, particularly the water extract of *E. purpurea*, while remaining ineffective against Rhinovirus replication, and other data suggest that *E. pallida* var. *angustifolia* may be more effective against Rhinovirus [7,8]. Multiple studies have shown that *Echinacea* augments the innate immune response, especially in the activation of macrophages (increased nitric oxide production, TNF-α, enhanced phagocytosis), dendritic cells (increased co-stimulatory molecules), and T-cells (increased cytokines) [9-13]. NK cytotoxicity has been shown to be enhanced with *Echinacea* treatment as well [14]. It has been suggested that *Echinacea* has a greater effect on alveolar macrophage response compared with splenocytes [15], a finding which may support the use of *Echinacea* for respiratory infection in particular. In addition to these immunostimulatory effects, *Echinacea* has also been shown to have an anti-inflammatory effect with the decreased production of IL-1β and TNFα [14] and decreased IL-6 in spleen cells [16].

Effects of different chemical constituents of *Echinacea* have been found to be active in modulating the immune response. The composition varies based on preparation type and part of the plant utilized. Polysaccharides are found in water extracts, whereas

alkamides are typically found in ethanol extracts. Caffeic acid derivatives and phenolic compounds are found in both water and ethanol preparations of *Echinacea* extracts [17]. Polysaccharides from *Echinacea* have been thought to influence the macrophage function and contribute to the direct anti-viral activity [7]. Alkamides and caffeic acid derivatives play a larger role in decreasing the effects of pro-inflammatory cytokines and providing an anti-inflammatory component [18,19]. Alkamides present in *Echinacea* extracts may be responsible for a decrease in NFk-B mediated inflammation [10]. Also, the phytochemical content may vary by *Echinacea* species. The varied chemical constituents and concentrations of these constituents found in different extracts likely contribute to the varying results that have been reported in the literature [20]. Given the variability in plant species and type of extract, along with the diverse possible mechanisms of action, the goal of this study was to systematically assess the multiple ways in which *Echinacea* may contribute to host defense against influenza infection using standardized water and ethanol extracts prepared from three species grown in defined conditions. The three species evaluated were *E. purpurea, E. angustifolia*, and *E. paradoxa var. paradoxa. E. angustifolia* and *E. purpurea* have been widely studied, whereas *E. paradoxa* var. *paradoxa,* used in this experiment, has not been as widely investigated for its medicinal properties. Given that *E. purpurea* and *E. angustifolia* have been commonly used, two different accessions of each of these species were tested to determine whether variability due to plant growing conditions could impact immunomodulatory response. The potential mechanisms by which *Echinacea* may contribute to host defense against influenza that were evaluated in this study were: 1) direct effects of *Echinacea* on influenza virus replication, 2) innate host defense at the initial site of influenza virus infection

(respiratory epithelial cells), and 3) immunomodulatory and anti-inflammatory action of *Echinacea* on host immune cell populations (spleen) under influenza-virus infected or non-infected conditions.

The initial hypotheses were that the three *Echinacea species* (both water and ethanol extracts) may have anti-viral effects, and the water extracts may increase the cytokine and chemokine response to influenza infection in spleen cells and A549 cells. In addition, the initial hypothesis predicted that the ethanol extracts would decrease cytokine and chemokine concentration, similar to previous findings of anti-inflammatory effects with ethanol extracts.

Materials and Methods:

Plant & Extract.

Echinacea angustifolia (Eang) (accessions PI631285 -*Eang1*, accession A28187-*Eang2*), *Echinacea purpurea (Epur)* (accession PI 631307 *Epur1*, accession PI633669 *Epur2*), and *Echinacea paradoxa* var. *paradox*a (*Epara*) (accession PI633663 *Epara*) plants were grown at the USDA-ARS Regional Plant Introduction Station (Ames, IA). Water extracts were prepared by boiling 6 g of dried root in 100 ml of endotoxin free water in an endotoxin free flask, and stirred for 1 hour at room temperature. Extracts were filtered using endotoxin free glass filter paper, centrifuged for 15 min at 10,000 rpm and pellets discarded before freeze drying. Extracts were diluted to 14.67 mg/ml of extract. The ethanol extracts were prepared from the dried ground root powder which was placed into a Whatman 43 × 123-mm cellulose extraction thimble (Whatman International Ltd., Maidstone, UK) with glass wool. The roots were refluxed with 250

mL of organic solvents, 100% ethanol, for 6 hours using a Soxhlet extraction device (Zhai et al 2007). The extract dried using an R-114 rotary evaporator (Brinkman Instruments, Westbury, NY) at <30°C under reduced pressure and re-suspended in 55% ethanol for *Eang*1 and *Epur1*, or 43% ethanol for *Eang*2, *Epur* 2 and *Epara*. These extracts were stored in concentration solution at -20°C until used in each experiment.

***In vitro* experiments.**

Anti-viral assay.

The anti-viral activity of the extracts was assessed using the Madin-Darby Canine Kidney cell line (MDCK). Cells were plated in 96-well plates (flat bottom) at $2x10^5$cells/ml suspended in complete DMEM (Dulbecco's Modified Eagle Medium DMEM + 10% fetal bovine serum HyClone Laboratories Inc, 1% PenStrep- penicillin/streptomycin -Gibco , 0.1% Gentimicin - Sigma, 1% fungizone or Amphotericin B Gibco) and allowed to become confluent. Cells were treated with high (1500µg/ml) and low (300µg/ml) concentrations of extract (*Eang*1 H2O, *Eang*2 H2O, *Epur*1 H2O, *Epur*2 H2O, *Epara* H2O, *Epur*1 EtOH, *Epur*2 EtOH, *Eang*1 EtOH, *Eang*2 EtOH, and *Epara* EtOH) prepared in post-inoculation media (DMEM +0.01% Bovine serum albumin , 1% PenStrep- penicillin/streptomycin -Gibco , 0.1% Gentamicin - Sigma, 1% fungizone or Amphotericin B -Gibco, and 0.11125%TPCK-treated Trypsin-Worthington Biomedical Corporation). Two extracts were tested per 96-well plate. Immediately after adding the extracts, 10µl of undiluted Influenza A/34/PR/8 (HA 1024, $5.5X10^6$ EID50) virus was added. Serial 1:10 dilutions of the virus were performed. Positive and negative controls were performed with each assay run (1 plate). Positive

control received virus only with no extracts or vehicle. Negative control received no virus or extract. Plates were incubated for 1 hour at 37°C in a 5% CO2 humidified incubator. Extracts and virus mix were removed from each sample by gentle pipetting using a multichannel pipette starting at the highest dilution to the lowest dilution to prevent cross contamination of the wells. Fresh post-inoculation media was added (100µl per well), and the plates were incubated at 37°C in a 5% CO2 humidified incubator. At 48 hours post infection, 50µl of the supernatants were transferred to a second 96-well plate (U-bottom) for viral titer analysis by hemagglutination testing. 50µl of a 0.05% dilution of rooster red blood cells was added to each well in the U bottom plate and incubated at room temp for at least 1 hour. Positive wells were indicated by lattice formation due to viral cross-linking of the red blood cells; negative wells were indicated by a red cell button at the bottom of the well. Positive and negative results for each well in the assay were recorded on a 96 well plate map and numerated. These quantifications were then evaluated using the Spearman-Karber method of calculating $TCID_{50}$: m=xk+d [0.5-(1/n)r] where xk is the highest dilution, d is the spacing between dilutions, n is number of replicates for each dilution, r is the number of negative responses, and $10^m=TCID_{50}$. Inverse log of this value was used for reporting anti-viral activity.

A549 Cell Culture.

A549 cells, a human type II pulmonary epithelial cell model, were cultured into 24-well plates at a concentration of $2x10^5$cells/ml. The cells were allowed to become confluent (24-48hrs post-plating) before extracts (*Eang*1 H2O, *Eang*2 H2O, *Epur*1 H2O, *Epur*2 H2O, *Epara* H2O, *Epur*1 EtOH, *Epur*2 EtOH, *Eang*2 EtOH, and *Epara* EtOH) diluted to 30mg/ml in complete FK media (ATCC) (FK plus 1% PenStrep-

penicillin/streptomycin, 0.1% Gentamicin - Sigma, 1% fungizone or Amphotericin B Gibco, 10% fetal bovine serum HyClone Laboratories Inc.) or Vehicle (H2O only or EtOH only - at the same dilution present in the extract) were applied to the cells for 2 hours at 37°C in a 5%CO2 humidified incubator. Extracts were removed by gentle pipetting to avoid disruption of the cell monolayer, and fresh media plus Influenza A/34/PR/8 (HA 4096, $EID_{50} = 10^9$) or (HA1024, $EID_{50}=5.5X10^6$) were added at a dilution of 1:100 and incubated at 37°C in a 5% CO2 humidified incubator for 1 hour with gentle rotation every 15 minutes to ensure equal viral distribution. Complete FK media was used to adjust the volume to 1ml in each well. Samples were incubated at 37°C in a 5%CO2 humidified incubator. Supernatants were collected 24 hours post infection and frozen at -20°C until further analysis.

***Ex vivo* experiments.**

In the experiments involving uninfected mice, spleens were collected from untreated BALB/c male mice. In the experiments with infected mice, untreated BALB/c male mice were infected via an intranasal route with 30 µl of $5.5X10^6$ EID50 (HAU1024) Influenza A/PR/8/34 H1N1 virus under isoflurane anesthesia. Spleens were harvested from uninfected mice, and at 9 days post-infection from the infected mice. Spleens were harvested in stomacher bags containing 5 ml of digestion media (RPMI with 2% fetal bovine serum (FBS) with collagenase D at a concentration of 1mg/ml). Spleen tissues were subjected to Seward Stomacher® (Port St. Lucie, FL) paddle blender. Spleens were processed through 0.4µ cell filters with digestion media. The plunger of a syringe was used to break up the remaining tissue through the filter. The filtrate was suspended in 10 ml of digestion media. Samples were centrifuged for 10 min at 1200 RPM, washed twice

with complete RPMI (cRPMI), and re-suspended in 5 ml of cRPMI (RPMI with Hepes, glutamate, sodium pyruvate, penicillin/streptomycin, fungizone, 2-mercaptoethanol and 10% FBS) without collagenase D. Samples were treated with 2 mls of ice cold 0.015M ammonium chloride red blood cell lysing agent for 10 min, washed in cRPMI and re-suspended in 5 ml of cRPMI. Cell counts were obtained using the Z™ series Coulter Counter® (Beckman-Coulter, Inc., Brea, CA). Cells were re-suspended in cRPMI at $2.5x10^6$cells/ml, and cultured into 96-well plates (100µl per well, 22 wells per spleen). On the same day as culture, four wells of each spleen were treated with of the following: H2O only, *Eang*1 H2O, *Eang*2 H2O, *Epur*1 H2O, *Epur*2 H2O, *Epara* H2O, EtOH only, *Epur*1 EtOH, *Epur*2 EtOH, *Eang*2 EtOH, and *Epara* EtOH. In the spleen cell experiment using cells obtained from virus-infected mice, two additional wells for each extract treated spleen were stimulated with 10µl of A/PR/8/34, H1N1 (HA=1024, EID_{50}=$5.5X10^6$) diluted to 100 HAU/ $1x10^6$ cells, and UV inactivated for 30 min, and two wells for each extract treated spleen received 10µl of media alone for negative control. Samples were incubated at 37°C in a 5%CO2 humidified incubator, and supernatants were collected at 48 hours and 96 hours post stimulation. Supernatants were frozen at -20°C until further analysis.

Multiplex Assay.

Supernatants were collected from A549 cell culture and analyzed for cytokine/chemokine expression. The Milliplex® microsphere (EMD Millipore Corporation, Billerica, MA) assay was used to analyze A549 or spleen supernatants for expression of different cytokines and chemokines. The assay was performed following manufacturer's instructions. The plate was read using Bio-plex® Suspension Array

System (Bio-Rad laboratories, Inc., Hercules, CA) instrumentation. Data was exported from Bio-plex® Manager to Microsoft® Excel (Microsoft, Redmond, WA) for further analysis.

Analysis.

Statistical analysis was performed using student T-test of independent samples comparing extracts to vehicle control with SPSS® software version 20 (IBM Corporation, Armonk, New York). Graphs were made using SigmaPlot® (Systat Software Inc., San Jose, CA).

Results:

In order to evaluate the anti-viral effect of the extracts, two different concentrations were analyzed. A low dose (300μg/ml) and a high dose (1500μg/ml) of each extract were analyzed to determine if the concentration of the extract would have an effect on the viral load. Under conditions with a low dose of H2O extracts (Figure 1), a trend towards increased anti-viral activity (decreased viral titer/ml) was observed in *Epur*2 H2O ($p=0.06$), *Eang*2 H2O ($p=0.1$) and *Epara* H2O ($p=0.08$) treatment compared to H2O controls. The high dose application of H2O extracts (Figure 1b) demonstrated significantly increased anti-viral activity only with *Epur2* H2O ($p<0.05$) compared to H2O controls, although there was a trend towards decreased viral titer/ml with *Epur*1 H2O ($p=0.1$) treatment. These data suggest that the *Epur* extracts have a modest increase in anti-viral activity *in vitro,* and reduced viral load by ~ 1 log. Ethanol extracts were also evaluated using the anti-viral assay. The low dose of EtOH extracts (Figure 1c) had no significant effect on the viral activity. The high dose of EtOH demonstrated increased

anti-viral activity with *Epur*1 EtOH ($p<0.05$), *Epur*2 EtOH ($p<0.05$), and trends toward a decrease in viral titer/ml with *Eang2* EtOH ($p=0.1$) and *Epara* EtOH ($p=0.09$) treatments compared to the EtOH contols. *Eang*1 EtOH was unavailable for testing at the high dose in this assay. At the high dose of extract, the Epur EtOH extracts resulted in nearly a 2 log reduction in viral load. These data suggest that the high dose of *Echinacea* extracts were more effective at decreasing the viral titer, although possibility that this same concentration could be attained in vivo remains to be established. By comparing these values to an EtOH control, the anti-viral activity of the extract was not a result of the EtOH present in the extract.

Extracts were applied to A549 lung epithelial cells to observe the change in cytokine/chemokines in absence of viral infection (Figure 2). The results in Table 1 show that Influenza A/PR/8/34 infection of A549 cells for 24 hours results in a significant increase in the production of chemokines and cytokines (IL-6, IL-8, TNFα, IFNγ, eotaxin, G-CSF, GM-CSF, IP-10, MCP-1, MIP1β, RANTES, and VEGF). Only *Eang1* and *Epur1* were evaluated for the potential effects on uninfected cells, and the results showed minimal effects with a reduction in IL-9 with H2O *Eang*1 treatment, but an increase in IL-8 and MCP-1 (Figure 2 and Table 1). The *Epur1* H2O extract decreased RANTES but increased VEGF. The ethanol extracts had little effect, although ethanol vehicle control resulted in a slight reduction of IL-6, IL-8, MCP-1, and RANTES. The only ethanal extract that altered chemokine production significantly was *Epur1* which increased MCP-1 to only a modest extent.

In contrast to the findings with uninfected A549 cells, when *Echinacea* extracts were incubated with A549 cells followed by infection with influenza virus, more

consistent effects of the extracts were observed. The H2O extracts from all three species increased eotaxin, GM-CSF, IL-8, MIP-1α, and MCP-1 to the greatest extent (~ 2 fold or greater), whereas IL-1β, TNFα, TNF-β, and IL-15 were modestly increased by *Echinacea* (Figure 3). Similarly, the A549 model was used to evaluate the effect of *Echinacea* EtOH extracts during influenza infection of lung cells (Figure 3). The EtOH vehicle control was compared to H2O vehicle control to determine whether the concentration of EtOH used had any effect on the A549 cells. There were no significant changes observed in the production of cytokines/chemokines in cells treated with EtOH compared to those treated with H2O vehicle. With respect to the EtOH extracts, pre-treatment of A549 cells with *E. purpurea, E. angustifolia*, and *E. paradoxa* resulted in a modest increase or trend towards an increase in IL-8 and IL-1β, with variable effects on eotaxin and IL-15. The effect of EtOH extracts was not of the same magnitude as H2O extracts, and EtOH extract effects were generally inconsistent.

To assess the effect of *Echinacea* extracts on an immune cell mixed populations, spleens from uninfected mice were cultured and treated with each extract (Figure 4). In this experiment the immunomodulatory potential of *Echinacea* was examined under conditions in which virus was not present. Overall, greater levels of cytokine or chemokine production were observed by spleen cells compared with A549 cells, as would be expected. Treatment with the H2O extracts from all species either significantly reduced ($p < 0.05$) or tended to decrease ($p < 0.10$) the inflammatory cytokines IL-1α, IL-1β, IL-6, and IL-12p40. However the *E. purpurea* H2O extracts increased IL-4, IL-5, and IL-17 which may suggest an effect on T helper cytokines. The EtOH extracts from all three species significantly decreased nearly all of the cytokines

and chemokines, and the results showed that *E. purpurea* treatment was associated with the strongest immunosuppressive effect. These findings with immune cell populations from uninfected mice show that EtOH extracts have consistent anti-inflammatory effects.

To assess the impact of viral infection and extract treatment on a mixed immune cell population, mice were infected *in vivo* with influenza virus, and on day 9 post-infection the splenocytes were cultured and treated with extracts, without virus present in the culture. The effect of the virus infection in the mouse without stimulation in the culture is shown in Table 2 (H2O and EtOH vehicle treatment only). When splenocytes were obtained from infected mice, it is apparent that the baseline (no *in vitro* stimulus) production of several cytokines and chemokines was increased relative to non-infected mice (IFN-γ, IL-1α, IL-6, IL-12p70, MCP-1, and a trend towards increased TNFα and MIP2). The effect of the addition of H2O extracts to the splenocytes is shown in Figure 5. In general, the H2O extract from all three species decreased the inflammatory factors MCP-1, IL-1α, IL-1β, IL-6, and IFN-γ. However, the H2O extracts increased the immunomodulatory cytokines IL-2, IL-4, and IL-10, although the increase was relatively modest and somewhat variable.

The EtOH extracts were also applied to splenocytes from infected mice without influenza stimulation *in vitro* (Figure 6). Treatment with all the extracts tested (*Epur*1, *Eang*2, *Epur*2, and *Epara* EtOH) decreased concentrations of G-CSF, GM-CSF, RANTES, IL-1α, IL-1β, IL-6, IFN-γ, IL-5, IL-10, and IL12p40 ($p<0.05$) compared to EtOH vehicle control. In addition, *Epur1*, *Epur2*, and *Epara* EtOH extracts decreased MIP-2, MCP-1, IL-2, and IL-15. These results were similar to the findings with spleen

cells obtained from uninfected mice in that EtOH extracts generally exhibit anti-inflammatory effects.

The use of spleen cultures from mice previously infected with influenza virus and re-stimulated with UV-inactivated influenza *in vitro* was a means of addressing the effect of *Echinacea* on influenza antigen-primed cytokine responses. This model provided some insight as to how *Echinacea* may influence adaptive immunity. The effects of UV inactivated virus on primed splenocyte cultures treated with vehicle control only (H2O and EtOH) are shown in Table 3. The addition of UV-inactivated virus to primed splenocytes resulted in increased production of IL-2, IL-4, IL-5, and IL-10. The effect of H2O extracts on UV-inactivated virus stimulated splenocytes from previously infected (primed) mice is shown in Figure 7. The addition of H2O extracts did not further enhance the production of the cytokines, although *E. purpurea* extracts tended to increase IL-2, IL-4, or IL-5, but reduced inflammatory factors IL-6, IFN-γ, and IP-10. The findings with respect to EtOH extracts revealed a pattern similar to previous results on splenocytes in that EtOH extracts from all species decreased both cytokine and chemokine response to UV-inactivated virus (Figure 8). One exception to this finding was that only one of the *E. angustifolia* extracts (*Eang2*) had less of an anti-inflammatory effect and instead actually increased IL-2 and IL-5. All cytokine and chemokine data with all spleen cell conditions are included in Table 2 and Table 3.

Discussion:

The results of this investigation demonstrate that *Echinacea* extracts have effects at multiple levels of defense against influenza virus infection including: modest anti-viral

activity, stimulation of innate host defense (chemokine response) by lung epithelial cells, and modulation of cytokine and chemokine response by host immune cells (both innate and adaptive immunity). In this study, we have shown that *Epur* H2O extracts demonstrate limited anti-viral activity *in vitro* at both the low and high doses tested, whereas *Echinacea* EtOH extracts demonstrated anti-viral activity in the high dose only. Although the extracts reduced viral load, the reduction was approximately a one log difference which may or may not be of a sufficient magnitude to have effects *in vivo*. Therefore, it will be essential to follow-up these experiments to determine *Echinacea* administration in vivo can limit viral replication. It will also be important to identify the constituents that may contribute to the antiviral effect, particularly in the species in which an effect was observed at both low and high concentrations (*E. purpurea*). Fractions containing polysaccharides and chicoric acid from *Echinacea* extracts have been shown to have anti-viral activity against influenza and herpes simplex virus [8]. In another study, chicoric acid has been shown to have anti-viral activity against herpes simplex virus [21] Chicoric acid has also been shown to have anti-viral activity *in vitro* against human immunodeficiency virus 1 [22]. While polysaccharides and chicoric acid are present in both water and ethanol extracts, water extracts contain a higher percentage due to the water-solubility of these compounds. Alkamides and ethanol fractions of *Echinacea* have been shown to have anti-viral activity against influenza, but not as robust as the chicoric acid and polysaccharide fractions [7]. Therefore, future studies are warranted to identify the components that may contribute to the antiviral effects, and establish whether the quantity of the antiviral component is sufficient to limit viral load *in vivo*.

The spleen cells served as model to investigate the effect of *Echinacea* on immune cells, and this question was addressed in the context of viral infection (previously infected primed mice), or without virus (uninfected mice). The findings revealed that the effect of *Echinacea* water extracts varied based on whether virus was or was not involved. When spleen cells were obtained from uninfected mice, H2O extracts from all species had some immunostimulatory effects including increased IL-10, IL-17, IL-5, IL-5, MIP-1α and MIP-1β. However when spleen cells were obtained from influenza-primed mice, modest increases in IL-10, IL-2, were found *with E. angustifolia, E. purpurea,* and *E. paradoxa*, but only *E. purpurea* extracts resulted in increased IL-5, IL-17, MIP-1α and MIP-1β. If UV-inactivated virus was added to re-stimulate antigen-specific spleen cells, the results showed that only *E. purpurea* resulted in increased IL-2, IL-4, and IL-5. Therefore, it is possible that the constituents responsible for immuno-stimulation may be found at a greater concentration in *E. purpurea* as compared to the other species. Another interesting finding related to the H2O extracts was that the extracts tended to decrease production of inflammation-associated cytokines IL-6, IL-1β, IFN-γ, regardless of whether spleen cells were obtained from uninfected or infected mice.

One finding observed in this study was that *E. purpurea* water extracts demonstrated an increase in cytokines involved in the differentiation of T-cell subtypes (IL2, IL4, IL5, and IL17) in the spleen cultures stimulated with UV-inactivated virus. IL-2 and IL-4 promote the proliferation and differentiation of T-cells [23]. IL-4 and IL5 are considered Th2 cytokines. Th2 responses enhance humoral immunity and could provide a benefit if this enhancement were to lead to increased anti-influenza specific antibody. Also, the increase in IL-4 may contribute to a decrease in cytotoxic T cell response and

promote the differentiation of B cells [24,25]. This shift may also contribute to change in the balance of Th1/Th2 responses and with a reduction in Th1 it is possible that a reduction in Th1 associated immunopathology may be decreased [26-28]. In further support of this possibility, it was observed that the H2O extracts decreased inflammatory cytokine production (IL-1β, IL-6) but enhanced the production of the anti-inflammatory cytokine IL-10. To our knowledge, these are the first results to demonstrate that IL-17 response was affected by *Echinacea* extracts in an influenza infection model. While the role of IL-17 is currently under investigation, increased IL-17 has been shown to be beneficial in preventing lethal influenza infections in humans during the 2009 pandemic [29]. IL-17 has also been proposed as an adjuvant in mucosal vaccines [30]. The enhanced modulation of the T-cell differentiation associated cytokines suggests that *E. purpurea* could alter the course of influenza infection and could provide a benefit for enhancing the immune response to a second influenza exposure an area of study that has not been explored with *Echinacea*.

The ethanol extracts clearly had different effects than water extracts on cultured splenocytes. In conditions in which cells were obtained from uninfected mice and in conditions in which cells were obtained from previously infected mice, EtOH extracts had the same effect, a consistent reduction of cytokines and chemokines. These decreases are similar to those observed in studies conducted in RAW 264.7 macrophage-like cell cultures [31]. Other studies have reported decreased pro-inflammatory cytokines in infection systems with *Echinacea* extracts prepared in ethanol [10,32], and therefore it is not surprising that the same anti-inflammatory effects were observed. The anti-inflammatory properties of ethanol extracts could have benefits in an influenza infection

model by limiting immune-mediated tissue damage. It will be important to identify the active components in future studies.

Another consistent finding observed in these experiments was that H2O extracts obtained from *E. purpurea, E. angustifolia,* and *E. paradoxa* stimulated chemokine production by A549 cells. However, the EtOH extracts had little impact on cytokine or chemokine production by A549 cells. The A549 model represents the type of cell that is first infected by influenza virus in the respiratory tract, and the possibility that *Echinacea* stimulates chemokine production would be expected to result in a more rapid recruitment of appropriate immune cells to the lungs. It has been established that lung epithelial cells secrete a variety of chemotactic proteins in response to infections. In the A549 culture, *Echinacea* water and ethanol extracts enhanced cytokines/chemokines responsible for cellular recruitment (Eotaxin, GM-CSF, IL-8, and MIP-1α/β). These chemokines have effects on specific cell populations in contributing to host defense. Eotaxin recruits eosinophils, and also a recent study by Sever-Chroneos et al demonstrated that increased GM-CSF expression in the lung could provide protection from influenza infection [33]. IL-8 is released from lung epithelial cells and induces neutrophil infiltration. While neutrophils are not the cells responsible for viral clearance, neutrophils may be more involved in wound healing after the resolution of infection. In one study, the removal of neutrophils had no deleterious effects on the outcome of influenza resolution [34]. Another study suggests that neutrophils can act as antigen presenting cells to effector CD8 cytotoxic T-cells [35]. MIP-1α and MIP-1β are involved in the recruitment of macrophages and monocytes. These chemokines may also enhance NK activity [36]. The increased chemokine production by lung epithelial cells could enhance the immune

response to influenza in the lung, and therefore H2O extracts of *Echinacea* may be useful during the first several days of infection when innate immunity is important.

One limitation with respect to the use of *Echinacea* is the high degree of variability in findings that has been reported in the literature. In this investigation, with a careful comparison of three different plant species using extracts prepared in an identical manner, it was possible to compare the effects of *Echinacea* on three relevant host defense responses important in influenza infection. The findings revealed that even with carefully controlled conditions, variations between species, type of extract, and plant accession are apparent. Findings from other studies also suggest that variation in the preparation techniques can yield changes in the immunomodulatory ability of the extract [37,38]. Also, slight variations within the same plant species in the relative change of response to influenza infection may be due to growing conditions from season to season [39]. To limit the variability as much as possible, the plants used for these experiments were cultivated and extracted by the Iowa State Botanical Center to ensure the extracts were as comparable as possible. In our study, *Eang1* (accession PI631285) and *Eang2* did not demonstrate the same effects of *Eang2* (accession A28187) and similarly with *Epur1* (accession PI 631307) and *Epur2* (accession PI633669). Each of these extracts was prepared from plants grown in different seasons. Also, *Eang1* and *Epur1* have been stored at -20°C for longer duration than *Eang2* and *Epur2* because of the different growth and cultivation year. In spite of the variability, there were consistent patterns that emerged. The H2O extracts from all species stimulated epithelial production of chemokines in response to influenza virus. Also consistent were the results showing that EtOH extracts had largely anti-inflammatory effects when cultured with spleen immune

cells. Of all three species studied, *E. purpurea* H2O extracts appeared to influence T helper cytokines to the greatest extent in influenza primed mice with an increase in IL-4, IL-5, possibly IL-17, but a reduction in anti-inflammatory cytokines. In future studies, it will be important to determine if *E. purpurea* H2O extract administered in vivo results in greater antibody production (resulting from greater IL-4 and IL-5), and limited lung inflammation.

To our knowledge, this is study is the first to evaluate *Echinacea* extracts in an anti-viral assay, lung model, and spleen immune cell populations. We have demonstrated that *Echinacea* water extracts demonstrate modest anti-viral activity *in vitro* independent of dosage, whereas anti-viral activity in *Echinacea* ethanol extracts was observed in the high dose only. *Echinacea* water and ethanol extracts enhanced cytokines/chemokines responsible for cellular recruitment in the lung, while the *Echinacea* ethanol extracts also provided some anti-inflammatory properties. While in the spleen *Echinacea* H2O extracts enhanced cytokine/chemokines for cellular recruitment and differentiation, and an anti-inflammatory profile was observed with the *Echinacea* EtOH extracts with variability seen between the species utilized. These data suggest that *Echinacea* may be beneficial in treating influenza infections. In conclusion, *Echinacea* modulated the influenza infection through direct anti-viral activity, enhancement of the lung epithelial and immune cell responses and decreased inflammation.

Chapter II figures.

Figure 1.

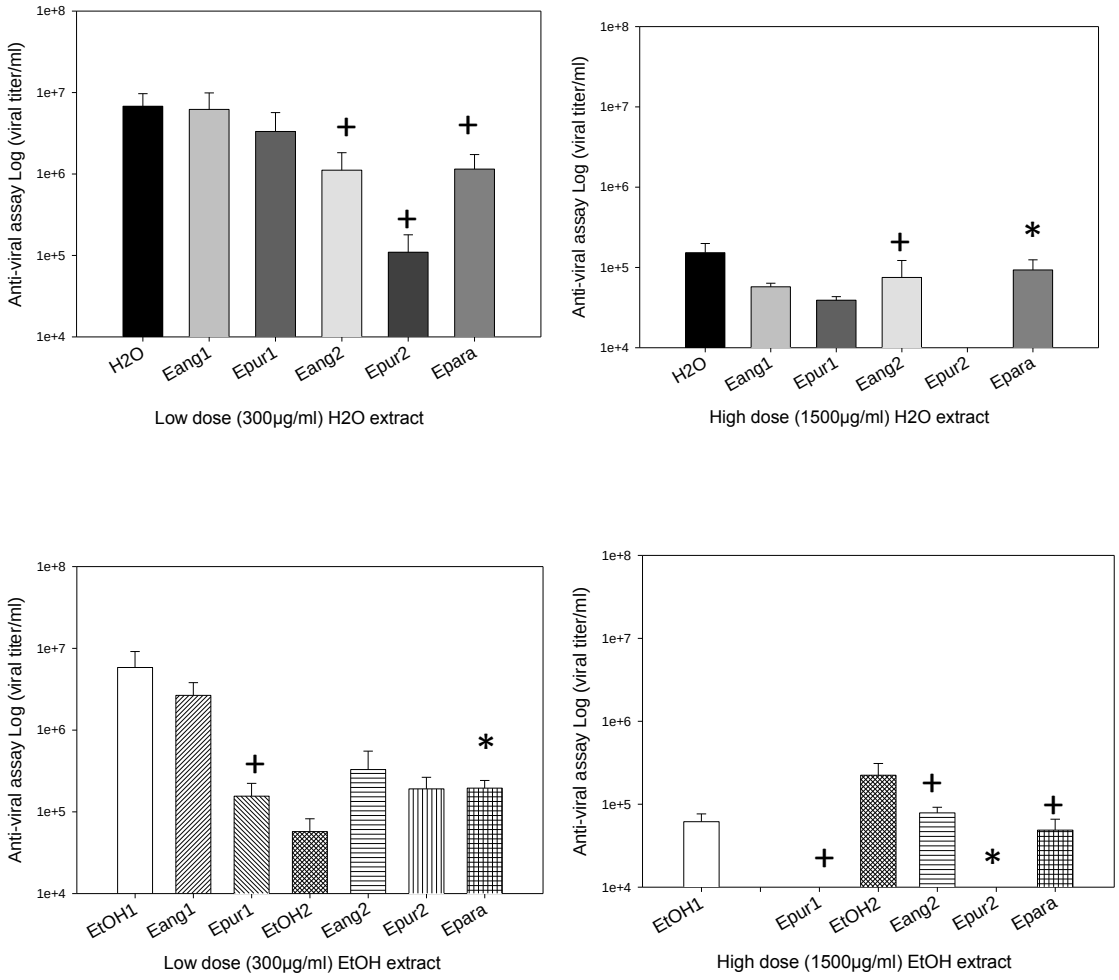

Figure 1: Anti-viral effect of extracts compared to vehicle controls using an anti-viral assay in MDCK cells. Extracts were applied to MDCK cells in low (300µg/ml) and high (1500µg/ml) dose concentrations. A/PR/8/34 was added in 1:10 dilution to 10^{-12}. Viral titers were calculated using Kirby-Spearmann calculations. Student t-test was utilized to determine statistical significance for each extract compared to the appropriate vehicle control (ex. H2O, EtOH1, EtOH2). Low dose water extracts *Eang2*, *Epur2*, and *Epara* demonstrated a trend towards decreased viral titer compared to the H2O control.

High dose water extracts *Epur1* (trend) and *Epur2* demonstrated a decrease in viral titer compared to the H2O control. Both low and high doses of EtOH extract *Epur1* showed a trend towards a decrease in viral titer compared to the EtOH1 control. Low dose of EtOH *Epara* was significantly increased compared to the EtOH2. Whereas, high doses of *Epur2* was significantly decreased compared to EtOH2 with *Eang2* and *Epara* demonstrating a trend towards a decrease in viral titer when applied in the high doses. n=4; *, $p<0.05$; + $p=0.1$.

Figure 2.

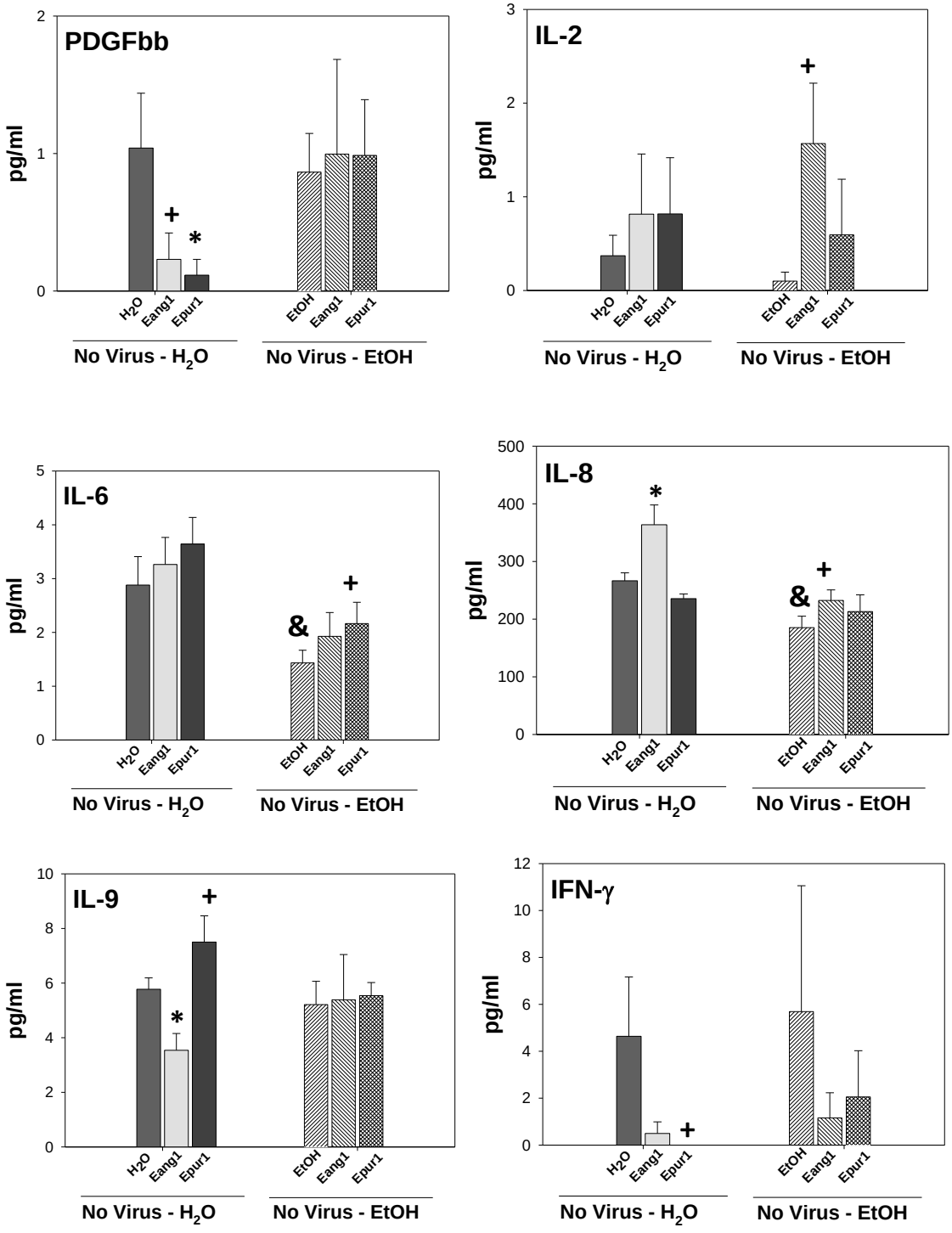
PDGFbb
pg/ml
2
1
0
+
*
H2O
Eang1
Epur1
EtOH
Eang1
Epur1
No Virus - H2O
No Virus - EtOH
IL-2
pg/ml
3
2
1
0
+
H2O
Eang1
Epur1
EtOH
Eang1
Epur1
No Virus - H2O
No Virus - EtOH
IL-6
pg/ml
5
4
3
2
1
0
&
+
H2O
Eang1
Epur1
EtOH
Eang1
Epur1
No Virus - H2O
No Virus - EtOH
IL-8
pg/ml
500
400
300
200
100
0
*
&
+
H2O
Eang1
Epur1
EtOH
Eang1
Epur1
No Virus - H2O
No Virus - EtOH
IL-9
pg/ml
10
8
6
4
2
0
*
+
H2O
Eang1
Epur1
EtOH
Eang1
Epur1
No Virus - H2O
No Virus - EtOH
IFN-γ
pg/ml
12
10
8
6
4
2
0
+
H2O
Eang1
Epur1
EtOH
Eang1
Epur1
No Virus - H2O
No Virus - EtOH

Figure 2. continued.

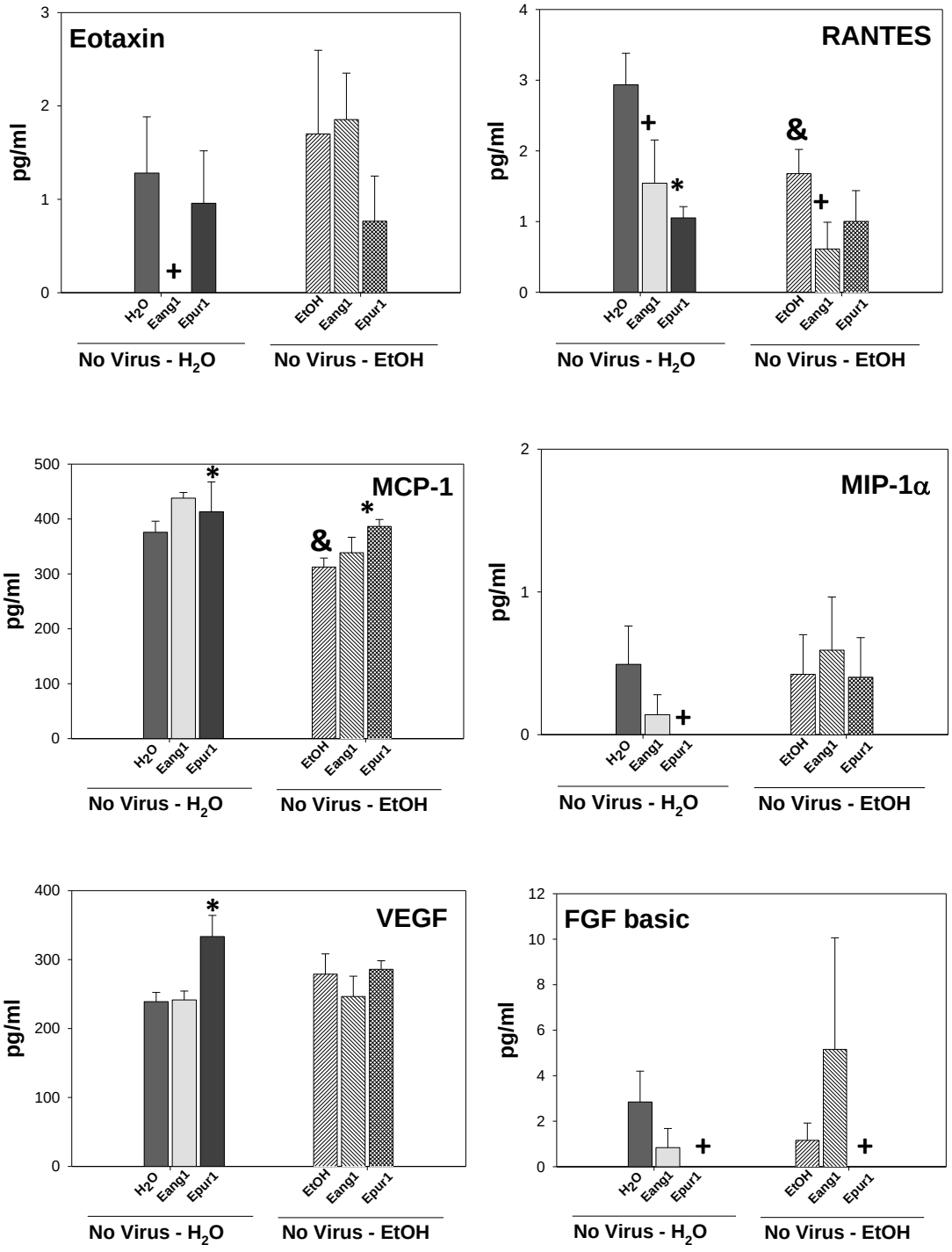

Figure 2: Effect of extracts on lung epithelial cells without virus present.

A549 cells treated with extracts and incubated without virus for 24 hours. Supernatants from the cells were analyzed using Bio-plex® multiplex assay. Student T-test was used to evaluate each extract and the appropriate vehicle control and the EtOH control against the water control. H2O= water, EtOH = ethanol, *Eang*1 = *E. angustifolia* extract 1, *Epur*1 = *E. purpurea* extract 1, *Eang*2 = *E. angustifolia* extract 2, *Epur*2 = *E. purpurea* extract 2, *Epara* = *E. paradoxa var*. *paradoxa* extract. H2O N=9; EtOH N=8; *Eang*1 and *Epur*1 EtOH N=5; *Eang*1 and *Epur*1 H2O N=4; *, $p < 0.05$; +, $p = 0.1$; & = $p < 0.05$ for EtOH vs. H2O control.

Figure 3.

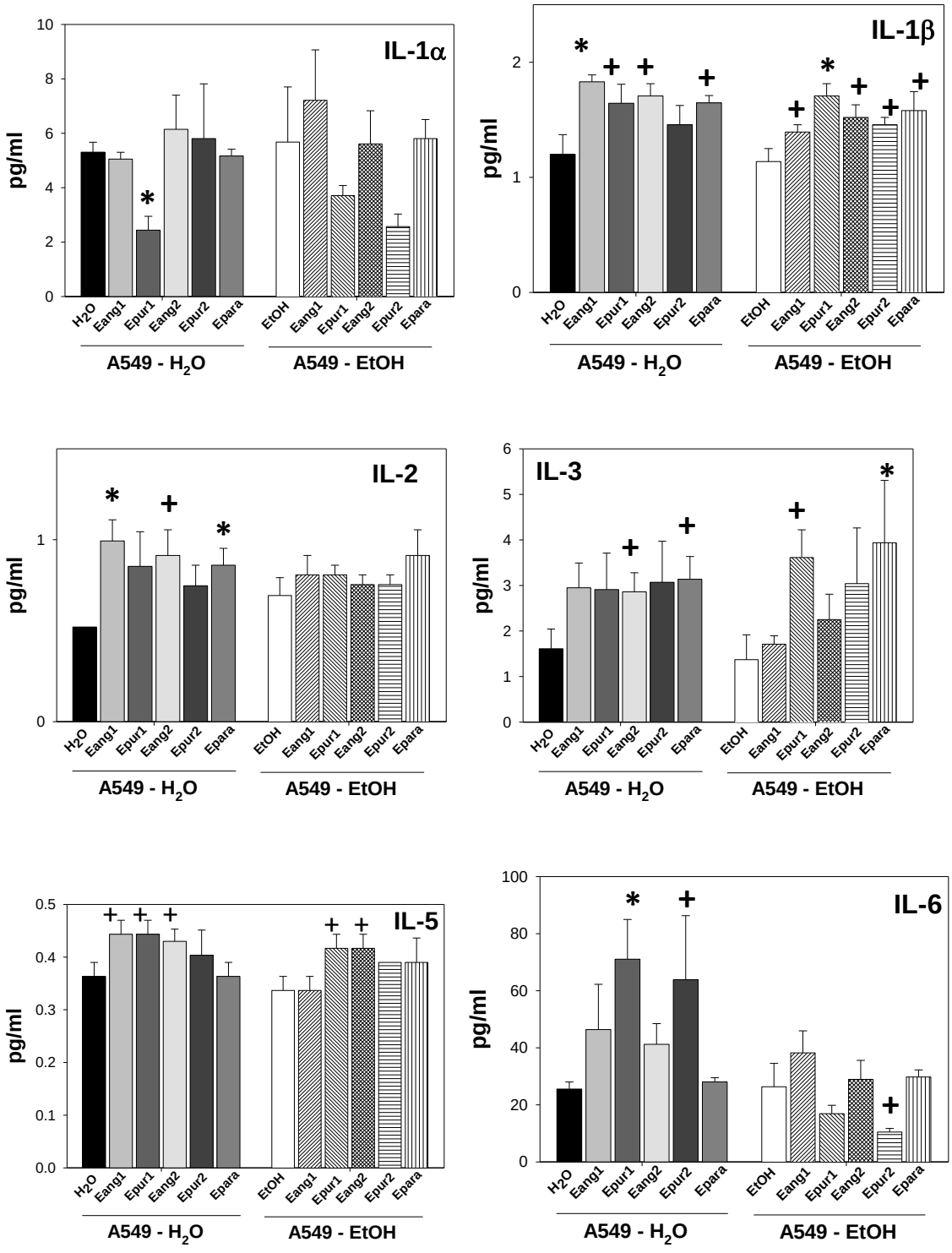
IL-1α
IL-1β
IL-2
IL-3
IL-5
IL-6
pg/ml
H2O
Eang1
Epur1
Eang2
Epur2
Epara
EtOH
A549 - H2O
A549 - EtOH

Figure 3. continued.

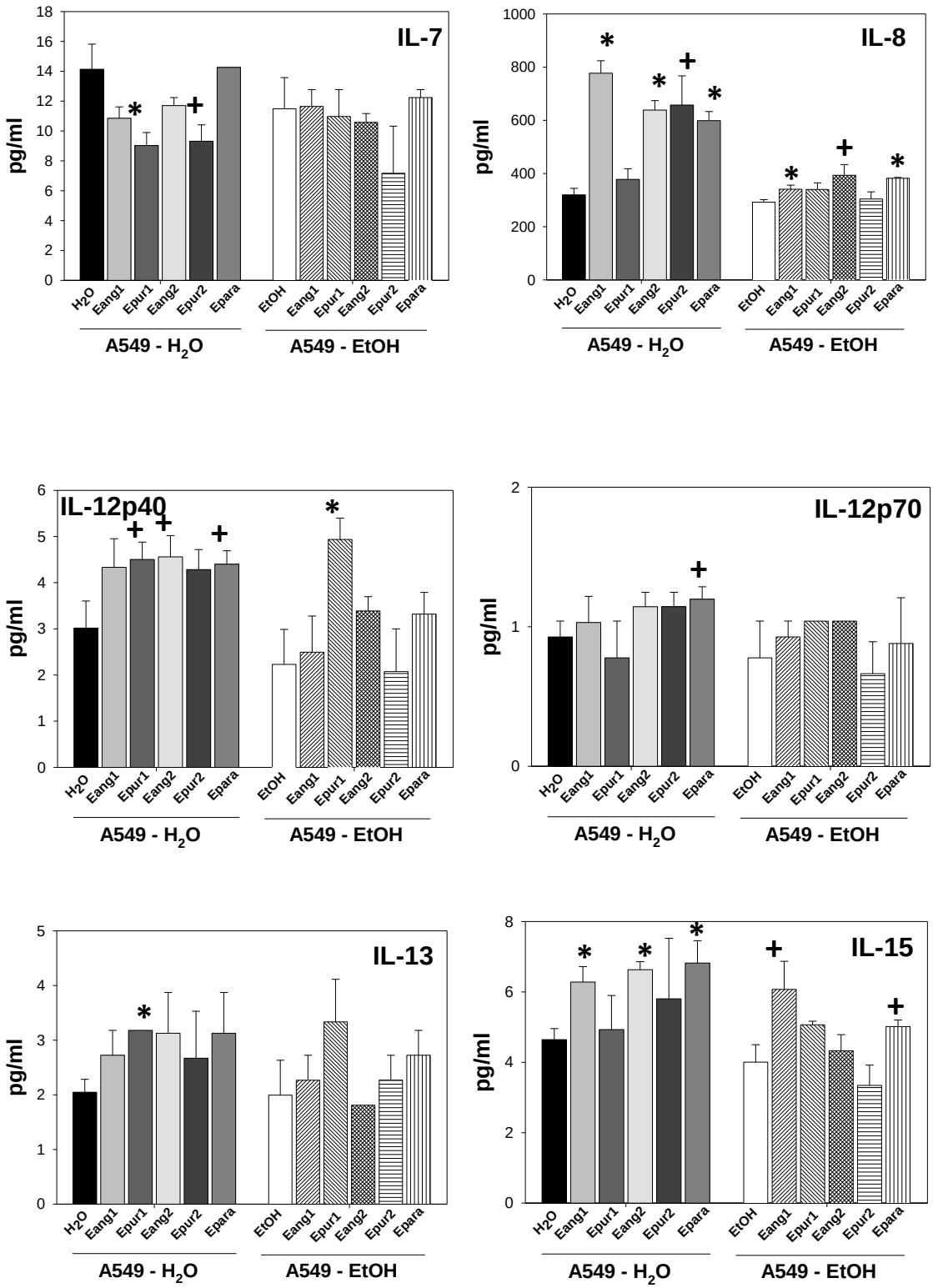

Figure 3. continued.

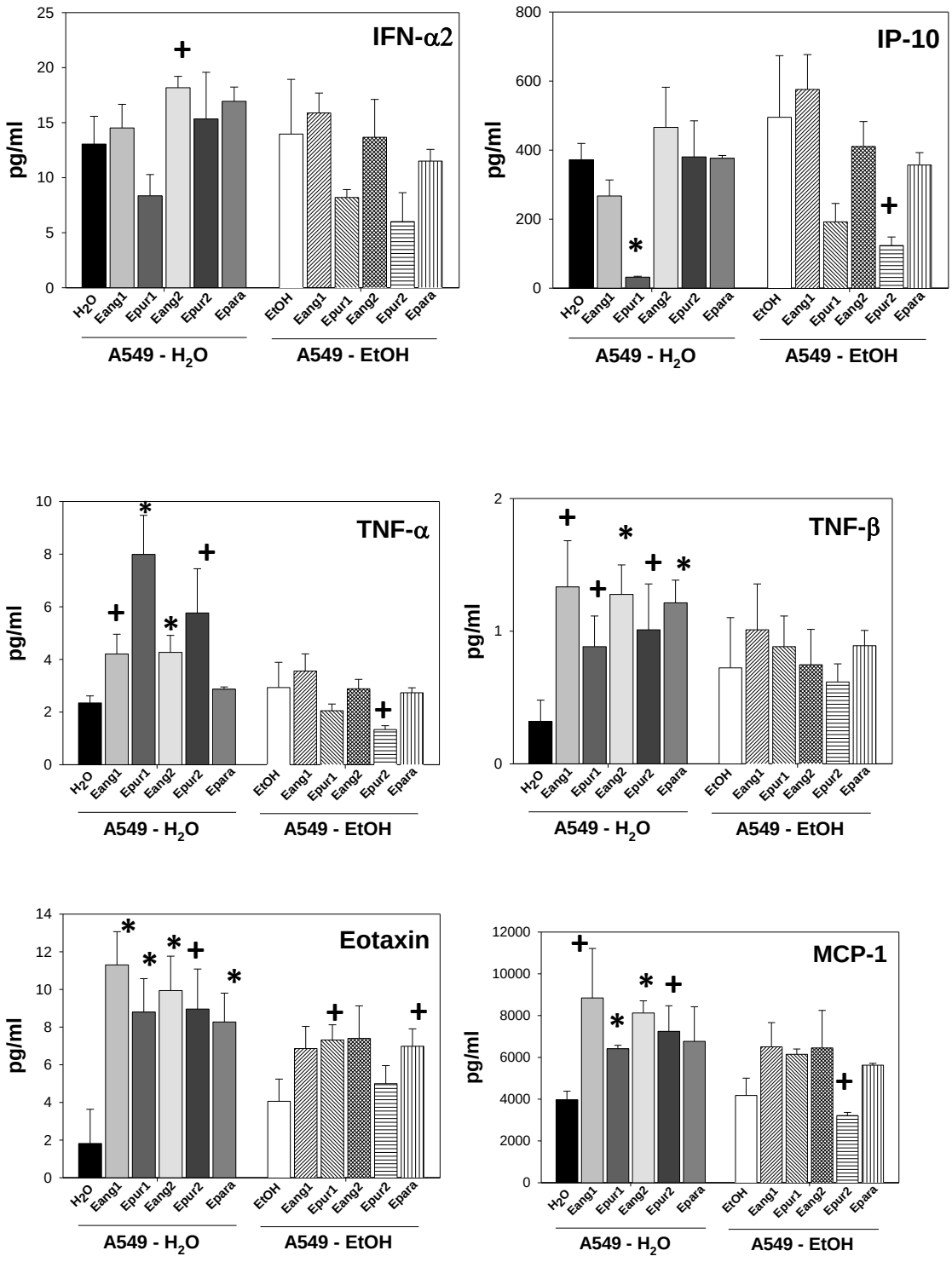

Figure 3. continued.

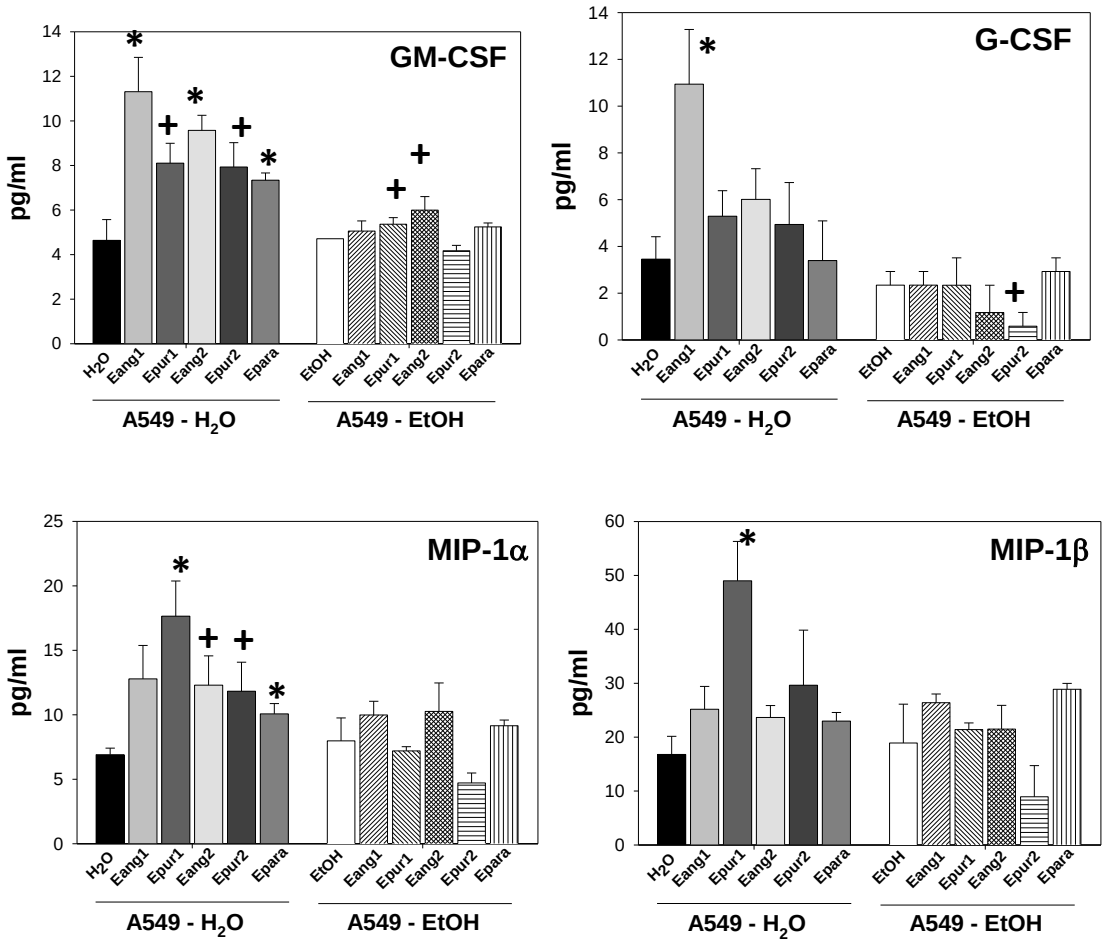

Figure 3: Effect of extracts on lung epithelial cells with influenza virus infection.

A549 cells treated with extracts and infected with Influenza A H1N1 PR/8/34 HA=4092. Supernatants were collected and tested using Bio-plex® multiplex assay. Student T-test was used to analyzed the extracts against the proper vehicle control (H2O extracts against H2O, EtOH extracts against EtOH). No significant changes between the EtOH vehicle control and H2O vehicle control were observed. H2O= water, EtOH = ethanol, *Eang*1 = *E. angustifolia* extract 1, *Epur*1 = *E. purpurea* extract 1, *Eang*2 = *E. angustifolia* extract

2, *Epur*2 = *E. purpurea* extract 2, *Epara = E. paradoxa var. paradoxa* extract. N=3 *, p<0.05; +, p<0.1.

Figure 4.

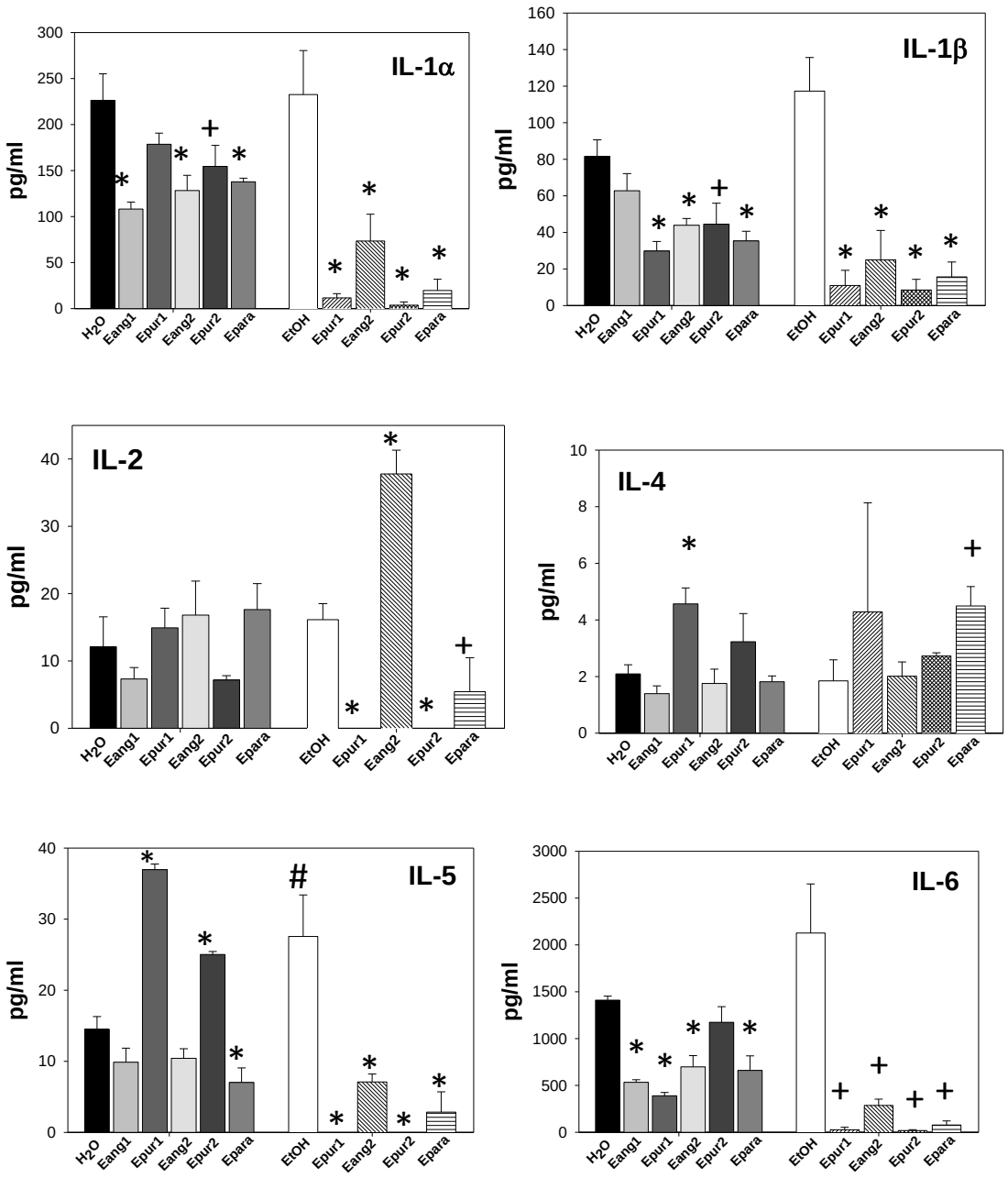

Figure 4. continued.

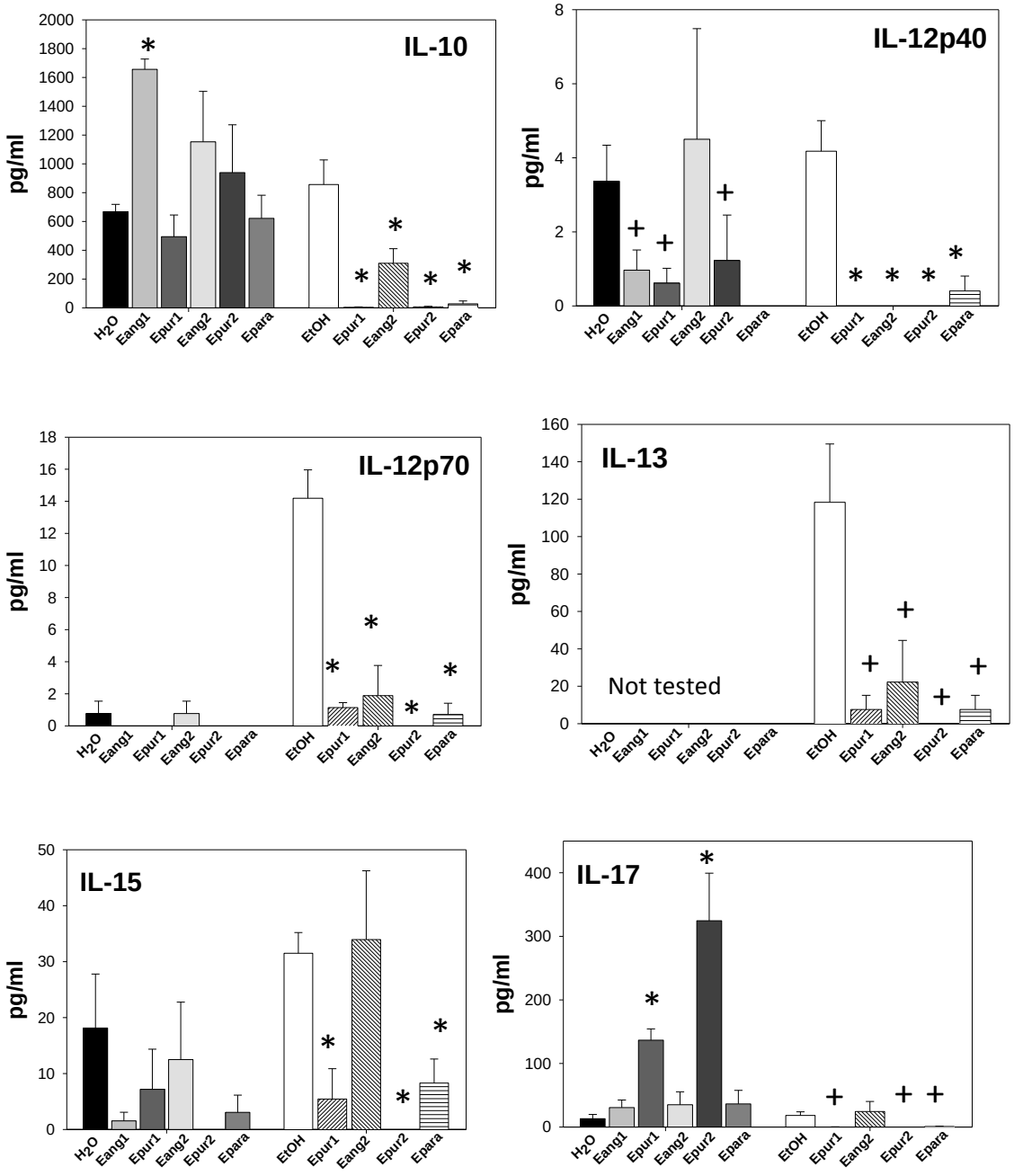

Figure 4. continued.

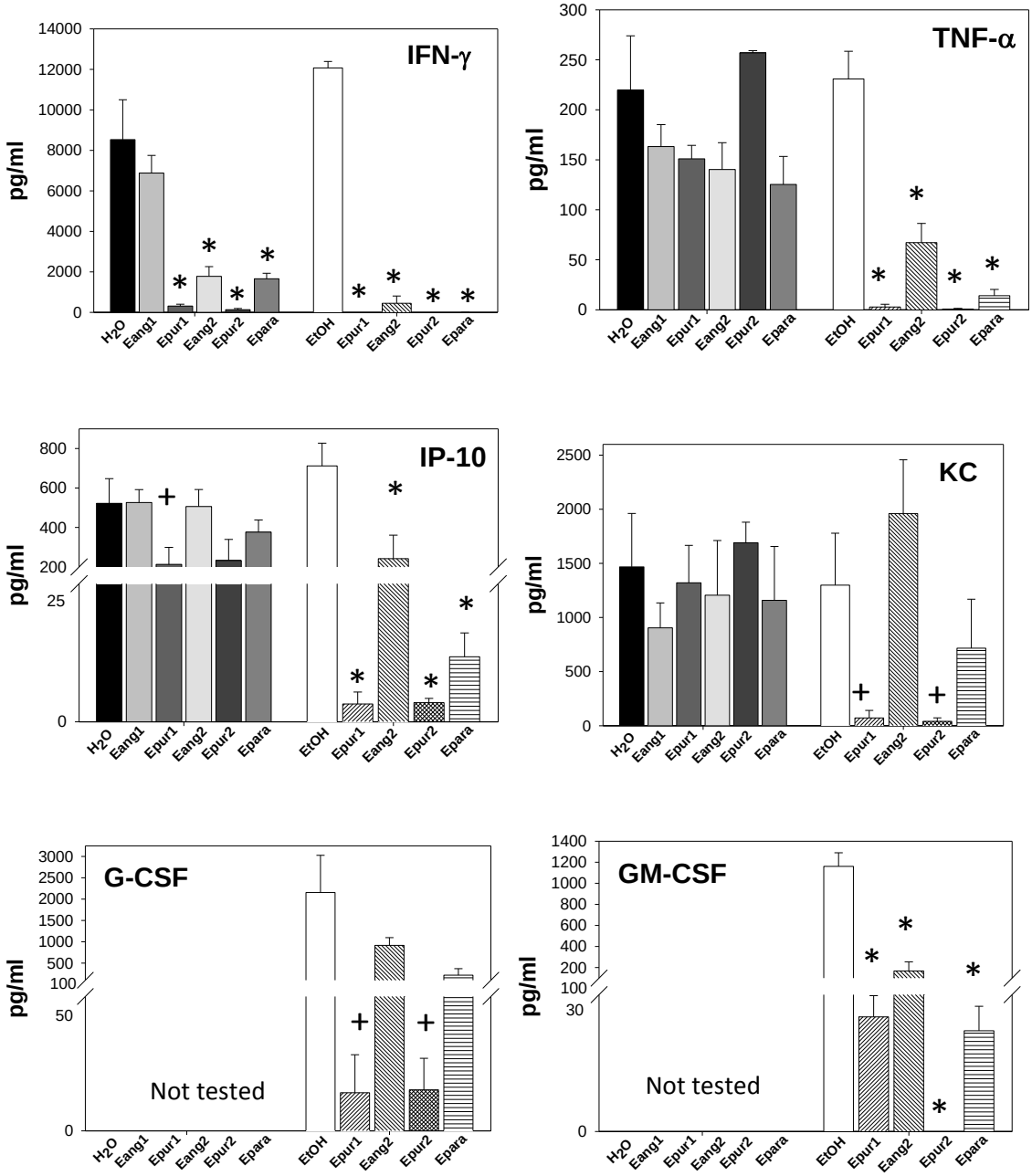

Figure 4. continued.

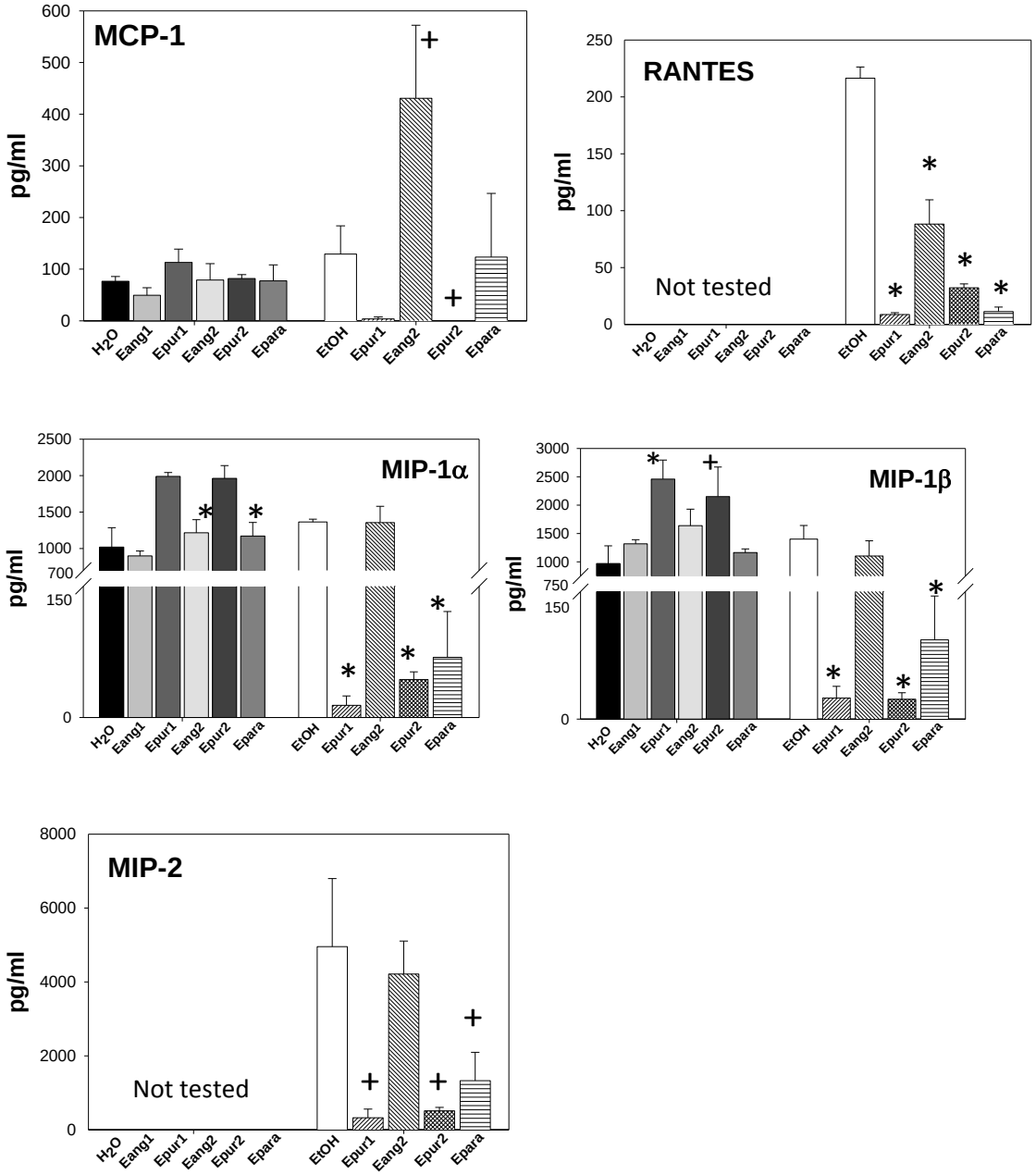

Figure 4: Effect of extract on splenocytes collected from uninfected mice.

Spleens were collected from uninfected mice, and splenocytes were cultured with each extract without virus. Supernatants were collected and tested using Bio-plex® multiplex

assay. Student t-test was used to compare the splenocytes receiving extract to vehicle control only (water extracts against water only; ethanol extracts against ethanol only). N=3, *, $p<0.05$; +, $p=0.1$. EtOH vehicle was also compared to H2O vehicle control. &, $p<0.05$; #, $p=0.1$.

Figure 5.

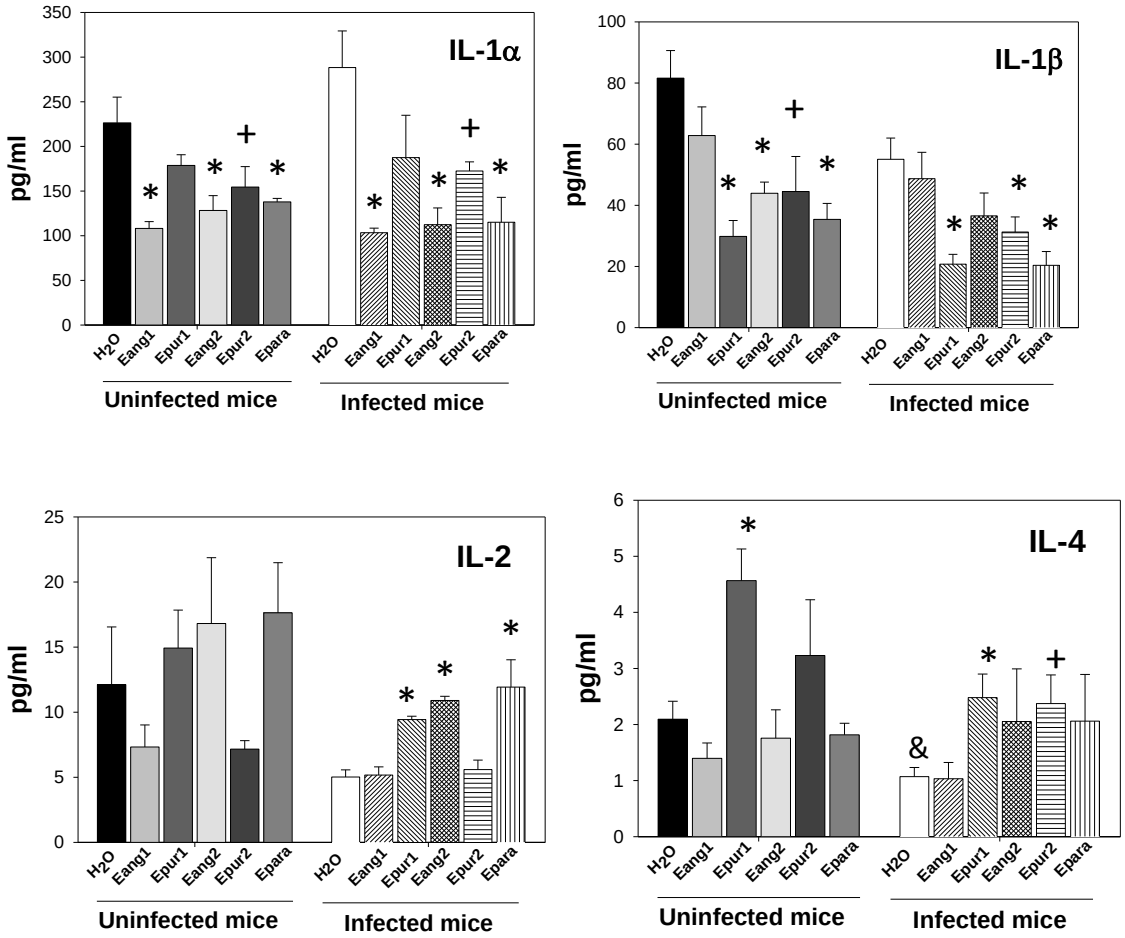

Figure 5. continued.

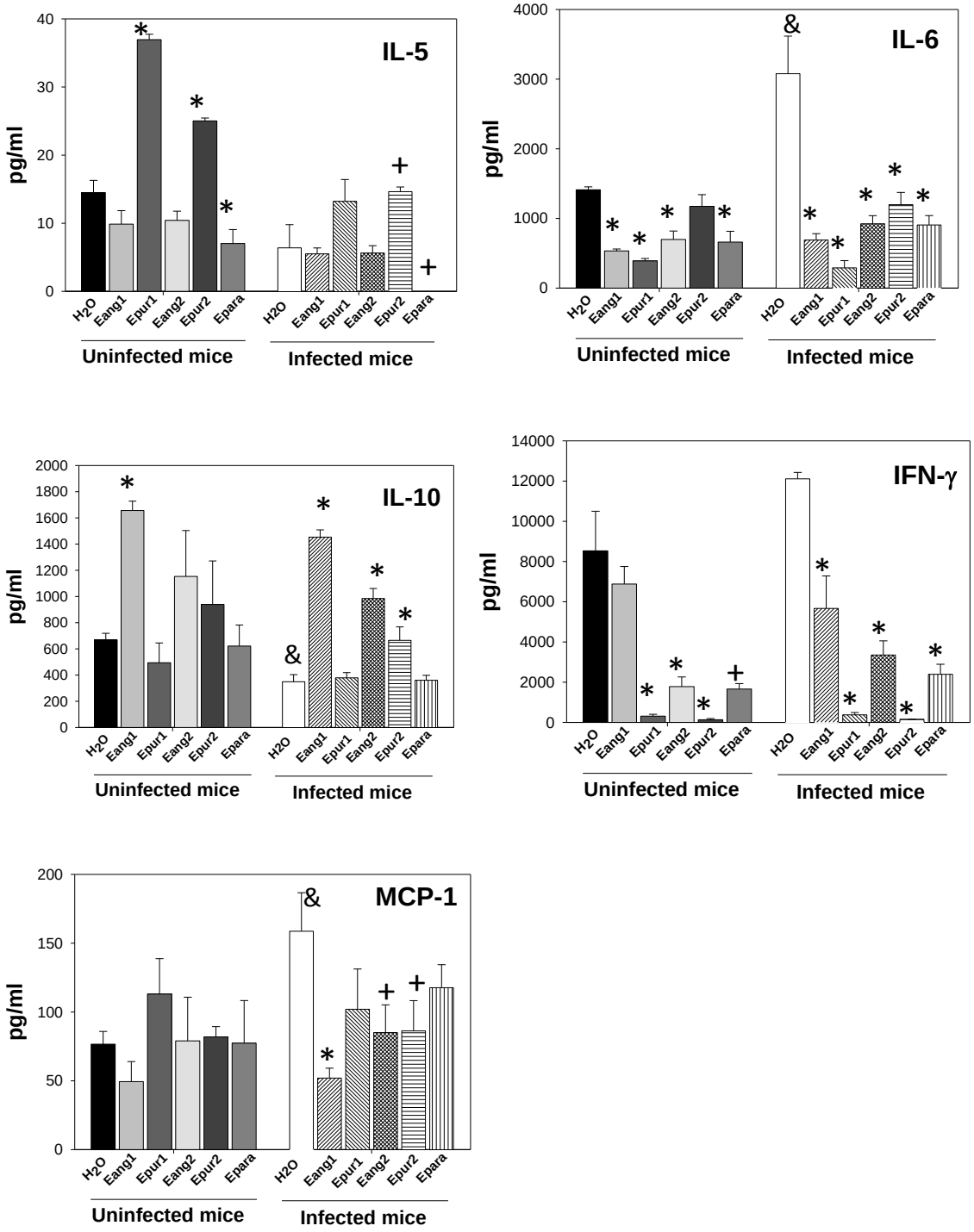

Figure 5: Effect of water (H2O) extracts applied to splenocytes from mice infected with influenza A/PR/8/34 compared to splenocytes from uninfected mice.

Cytokines/Chemokines were measured using Bio-plex® multiplex assay. Student T-test was used to determine significance of each extract compared to the appropriate water control. N=3; *, $p<0.05$; +, $p=0.1$. The effect of infection in the mouse was evaluated with the student t-test (comparing uninfected H2O control to infected H2O control). &, $p<0.05$, #, $p=0.1$.

Figure 6.

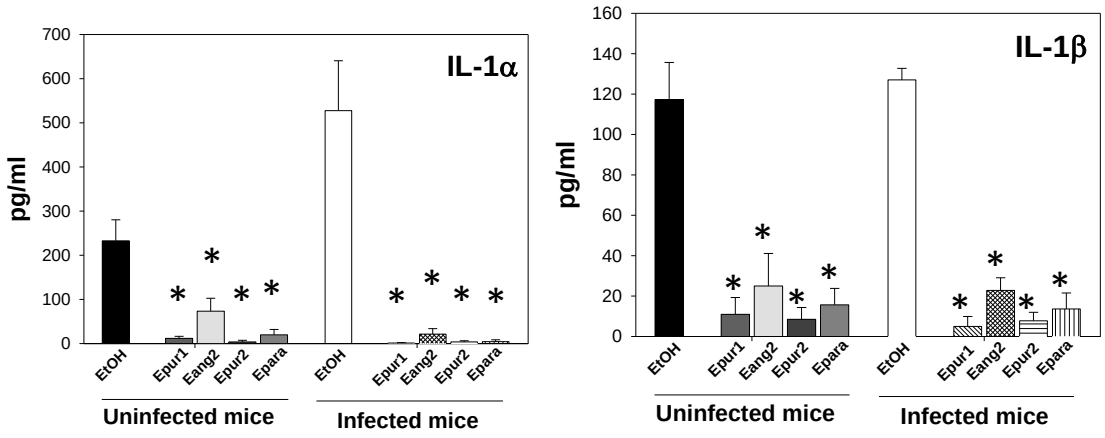

Figure 6. continued.

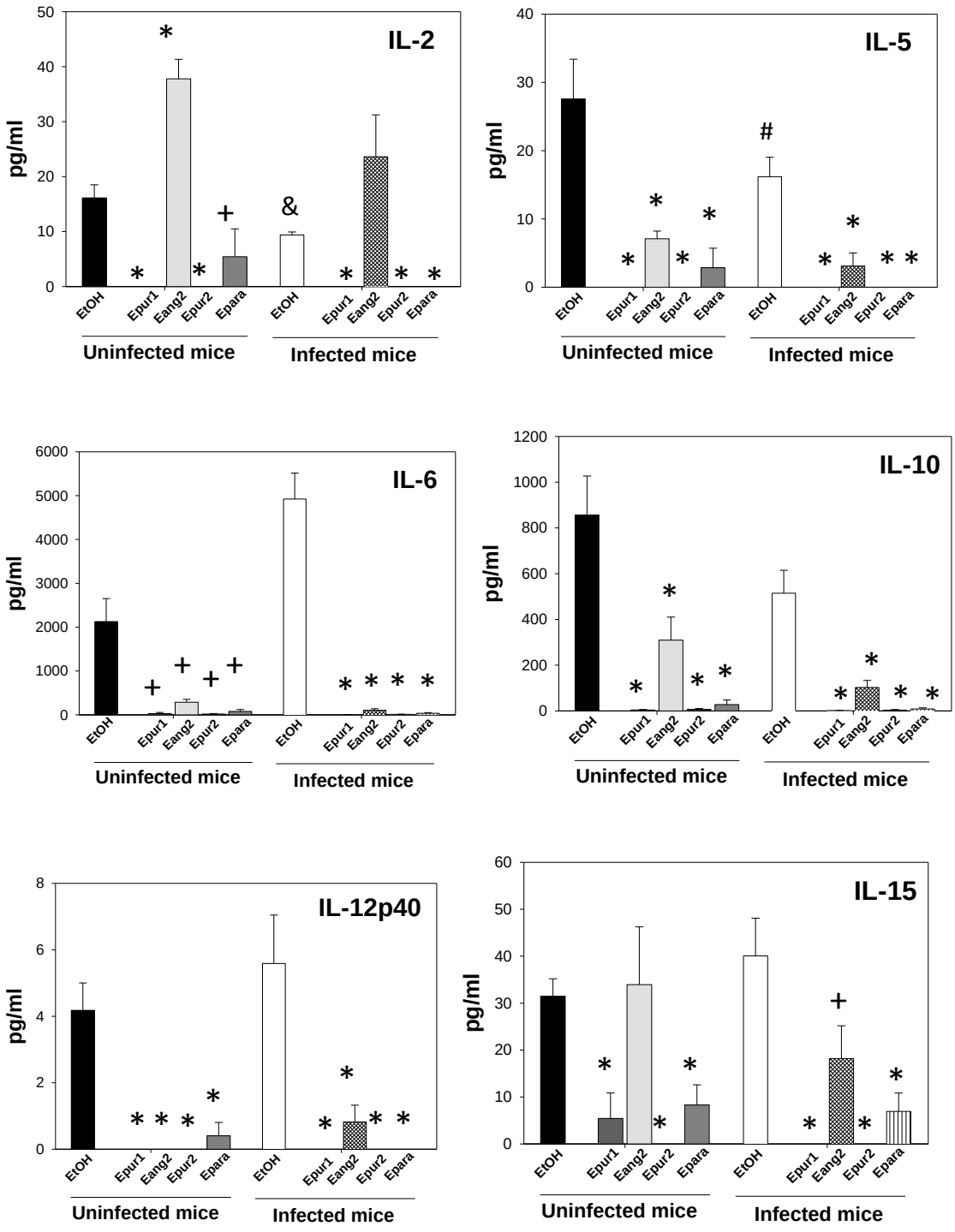

Figure 6. continued.

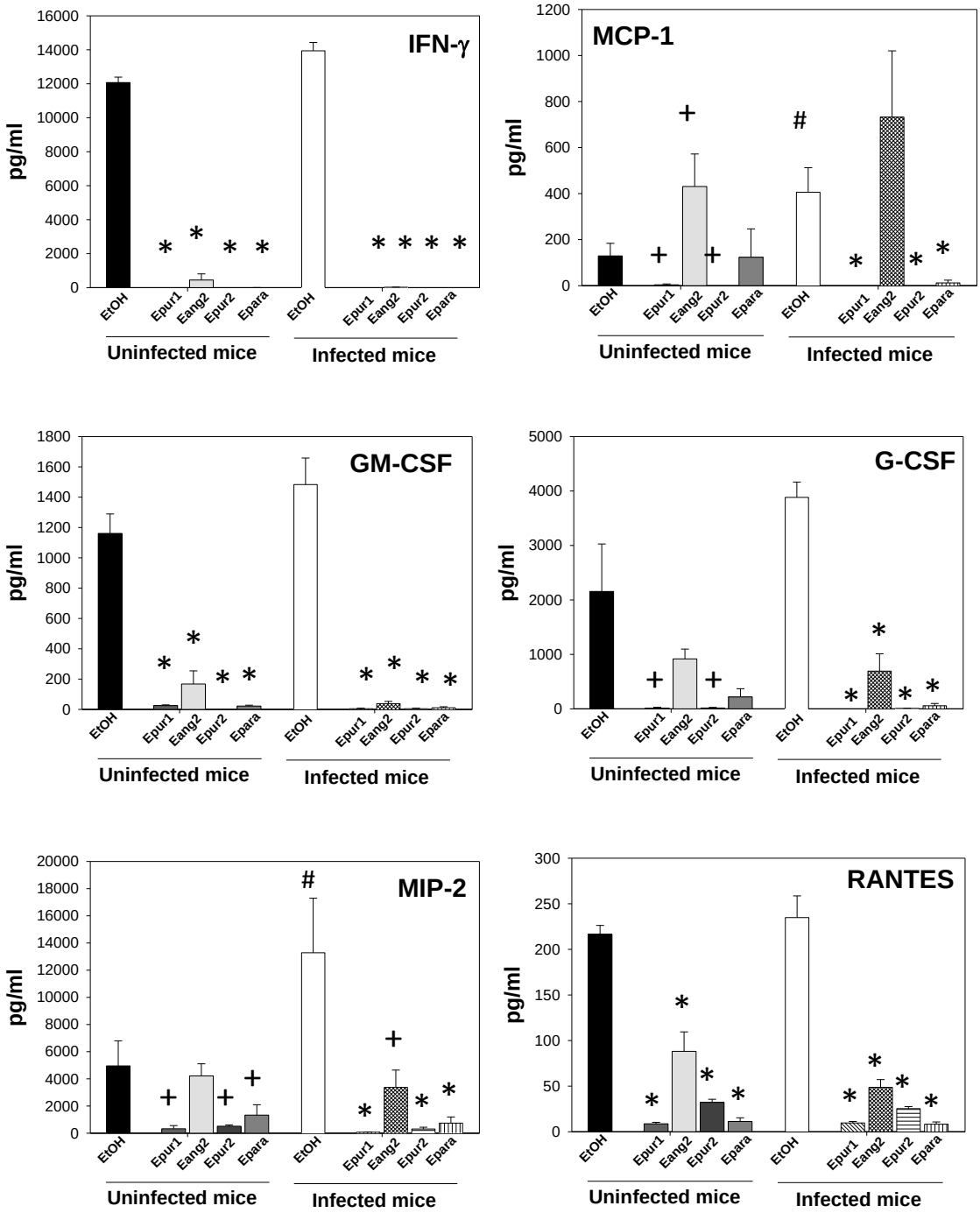

Figure 6: Effect of ethanol (EtOH) extracts applied to splenocytes from mice infected with influenza A/PR/8/34 compared to splenocytes from uninfected mice. Cytokines/Chemokines were measured using Bio-plex® multiplex assay. Student T-test was used to determine significance of each extract compared to the appropriate EtOH control. Uninfected mice N=3; infected mice N=4; *, $p<0.05$; +, $p=0.1$. The effect of infection in the mouse was evaluated with the student t-test (comparing uninfected EtOH control to infected EtOH control). &, $p<0.05$, #, $p=0.1$.

Figure 7.

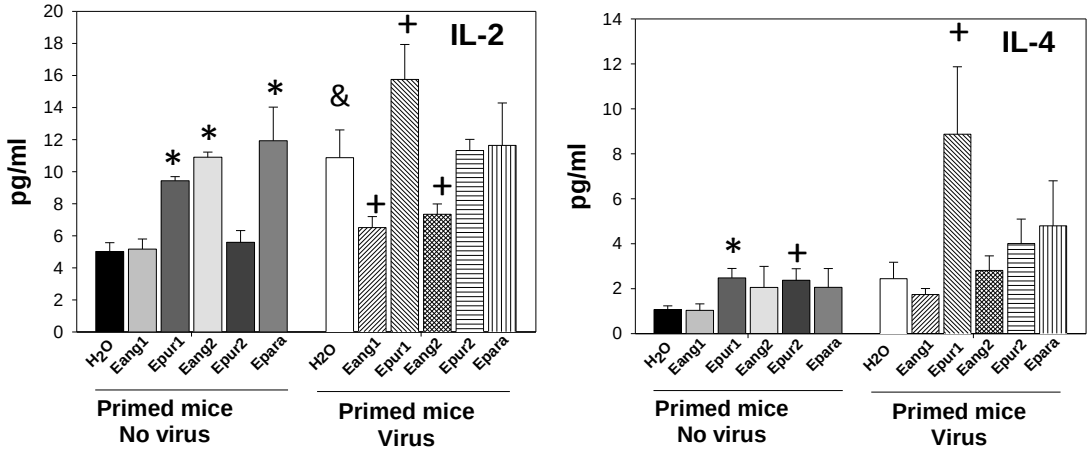

Figure 7. continued.

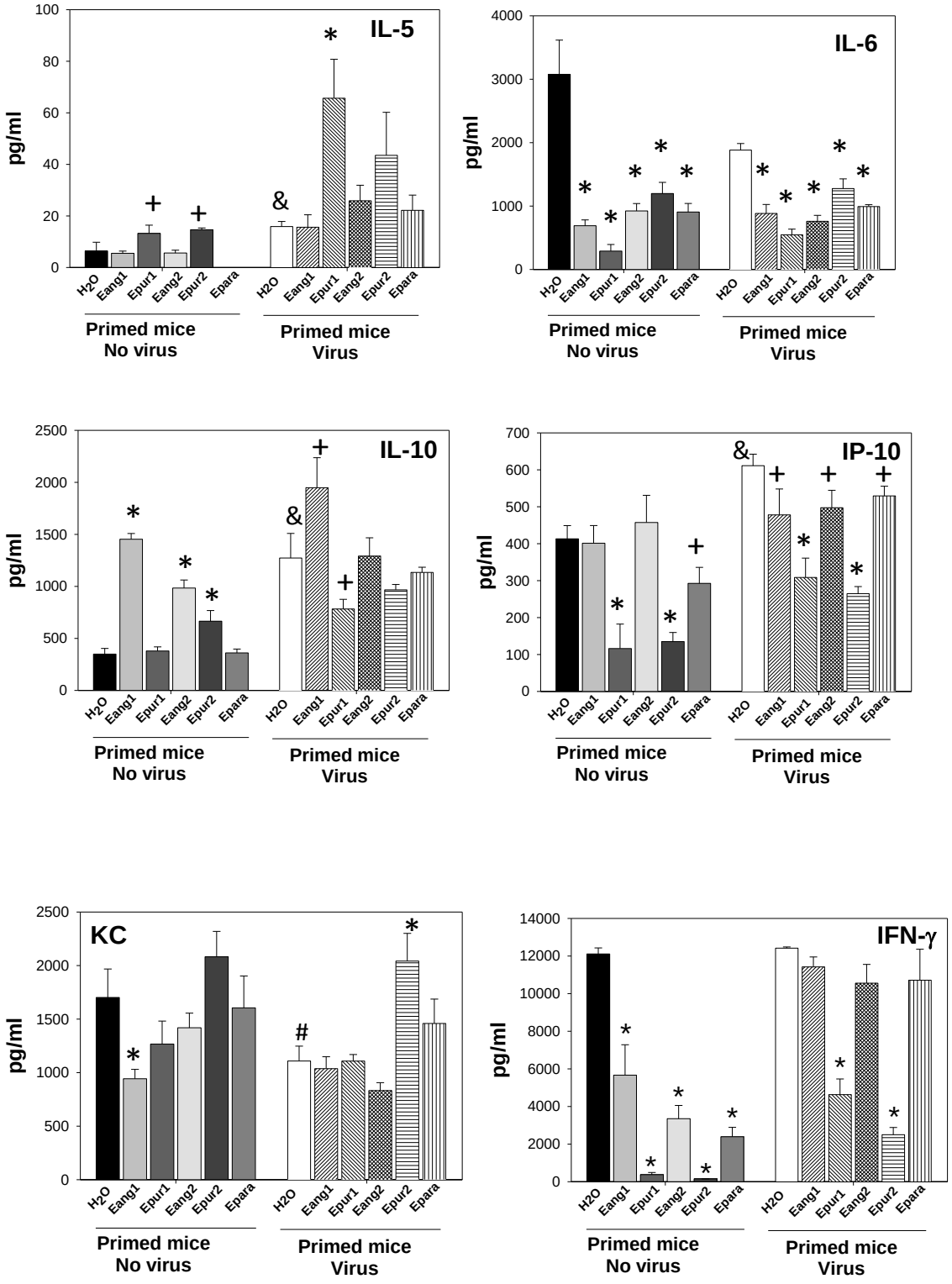

Figure 7: Effect of water extracts on splenocytes from mice primed with A/PR/8/34 and stimulated with UV inactivated H1N1 A/PR/8/34. Cytokines/Chemokines were measured using Bio-plex® multiplex assay. Student T-test was utilized to evaluate each extract against the appropriate vehicle control. Primed mice no virus N=3; Primed mice virus N=4; *, $p<0.05$; +, $p=0.1$. The effect of UV inactivated stimulation on primed splenocyte cultures was evaluated with the student t-test (comparing primed mice no virus H2O control to primed mice virus H2O control). &, $p<0.05$, #, $p<0.1$.

Figure 8.

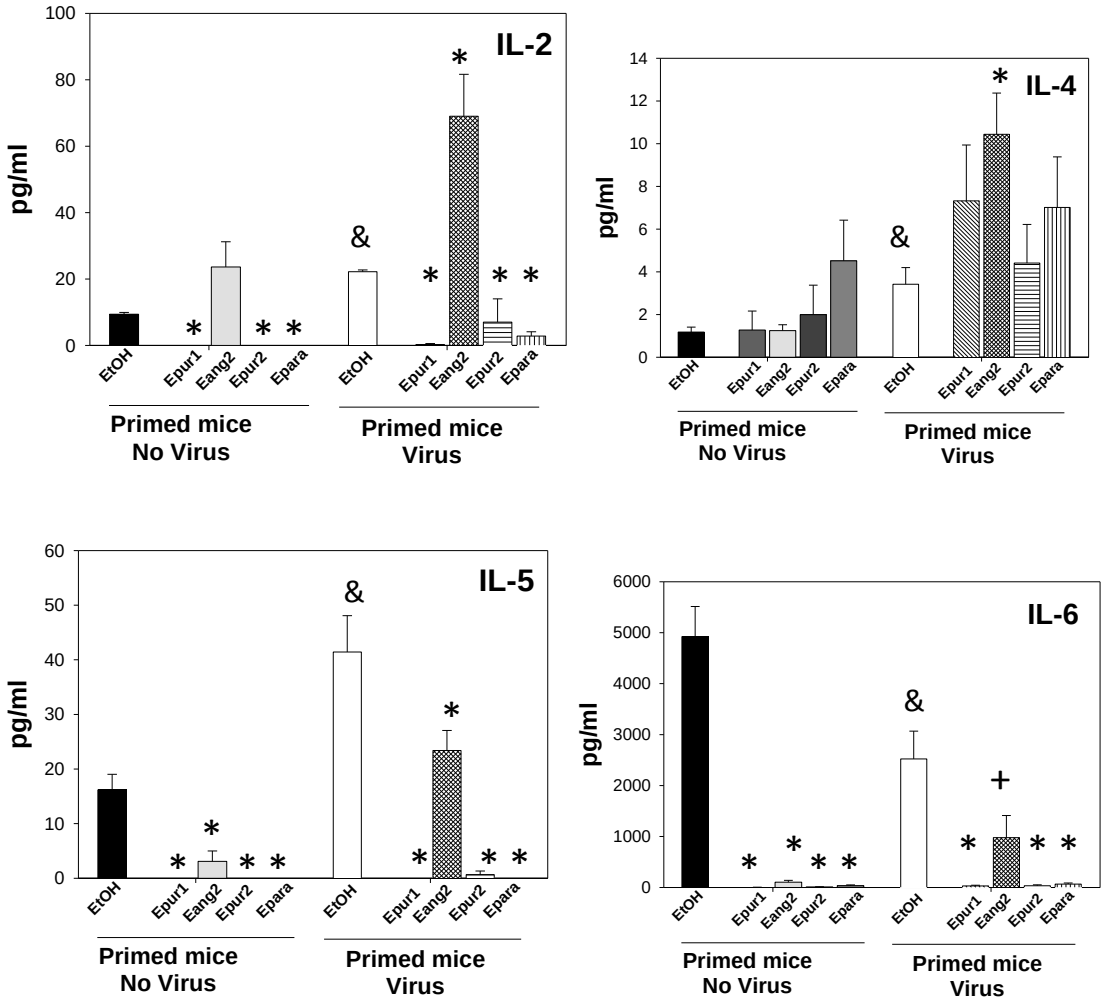

Figure 8. continued.

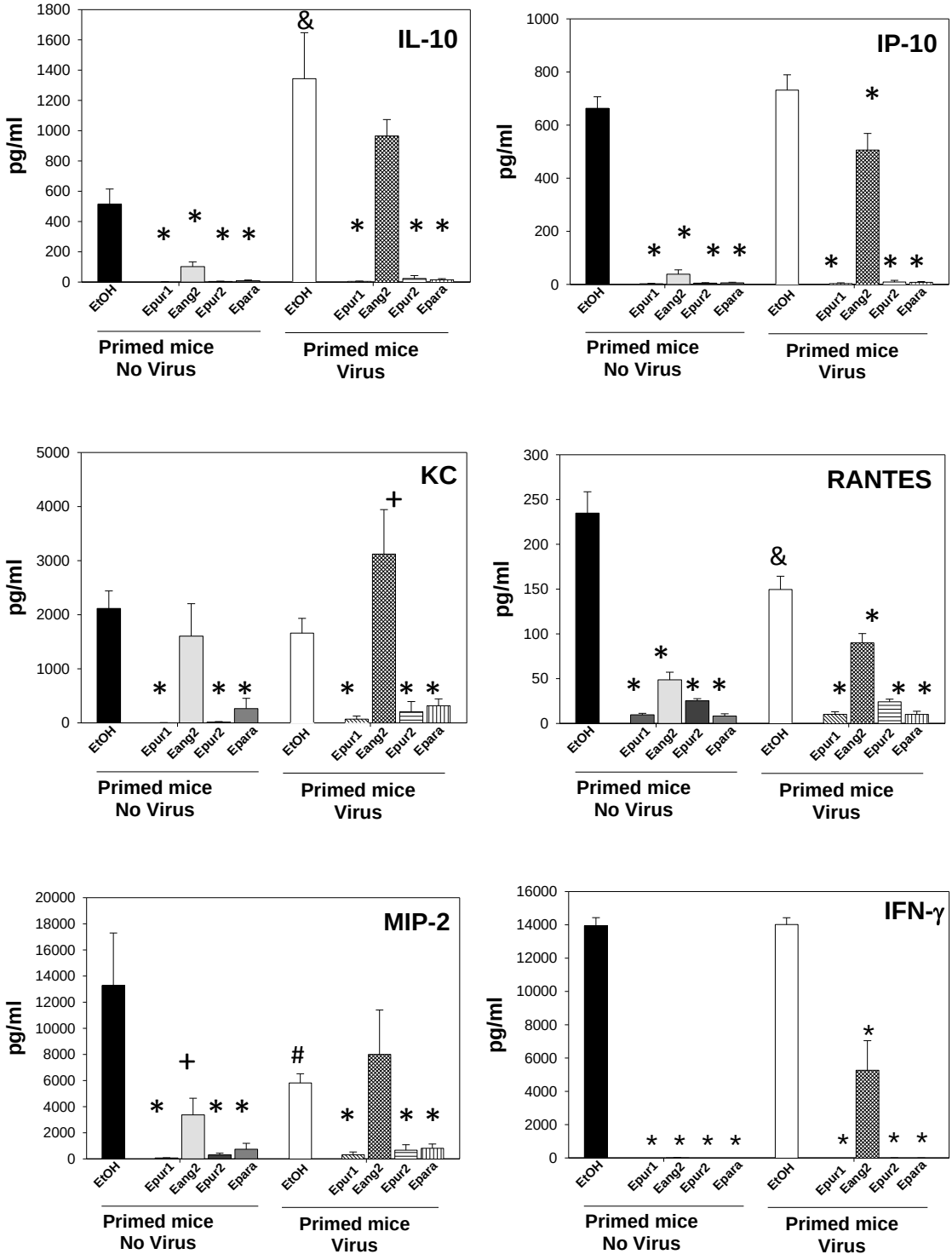

Figure 8. continued.

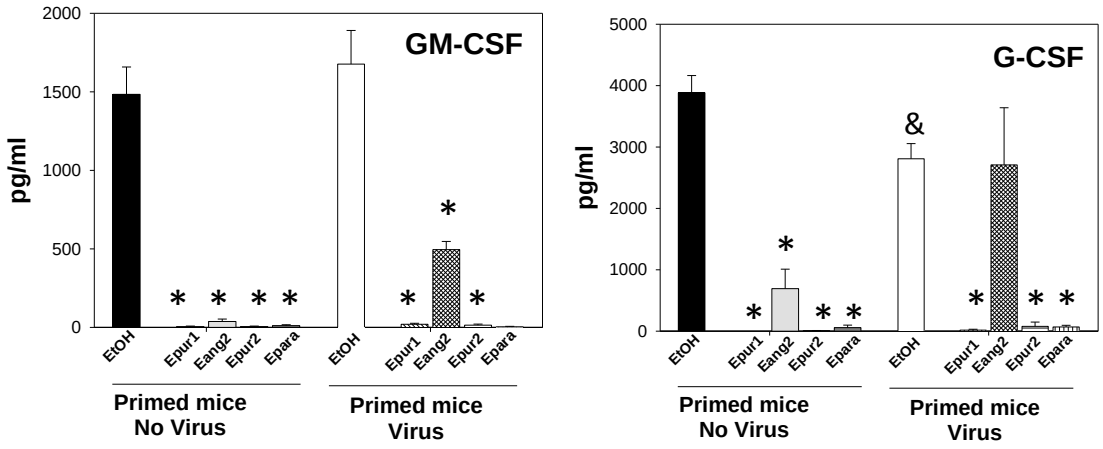

Figure 8: Effect of ethanol extracts on splenocytes from mice primed with A/PR/8/34 and stimulated with UV inactivated H1N1 A/PR/8/34.

Cytokines/Chemokines were measured using Bio-plex® multiplex assay. Student T-test was utilized to evaluate each extract against the appropriate vehicle control. Primed mice no virus N=4; Primed mice virus N=5; *,$p<0.05$; +, $p=0.1$ The effect of UV inactivated stimulation on primed splenocyte cultures was evaluated with the student t-test (comparing primed mice no virus EtOH control to primed mice virus EtOH control). &, $p<0.05$, #, $p<0.1$.

Table 1. Effect of virus infection on A549 cell cytokine/chemokine response.

	H2O					EtOH				
	no virus		virus		Virus vs no virus	no virus		virus		Virus vs no virus
n=	9		9			8		9		
	mean	std. error	mean	std. error	p-value	mean	std. error	mean	std. error	p-value
IL-1b	0.08	0.04	0.16	0.08	0.40	0.07	0.03	0.54	0.3	0.15
IL-1ra	0.58	0.38	0.58	0.38	1.00	0.18	0.12	1.36	0.84	0.20
IL-2	0.37	0.22	0.91	0.63	0.43	0.1	0.1	0.54	0.44	0.37
IL-4	0.01	0.01	0.61	0.14	0.00	0	0	0.7	0.15	0.00
IL-5	0.05	0.03	0.05	0.02	0.98	0.05	0.02	0.04	0.02	0.87
IL-6	2.88	0.53	350.75	21.39	0.00	1.43	0.24	415.43	34.74	0.00
IL-7	0.13	0.05	0.72	0.12	0.00	0.18	0.08	1.05	0.19	0.00
IL-8	266.55	13.74	1139.7	367.83	0.05	185.18	19.86	1007.5	289.77	0.02
IL-9	5.77	0.42	9.36	1.08	0.01	5.21	0.85	10.04	0.88	0.00
IL-10	0.2	0.1	0.3	0.15	0.56	0.23	0.12	0.27	0.12	0.79
IL-12p70	7.63	0.44	9.7	0.36	0.00	8.19	0.97	11.22	1.23	0.08
IL-13	0	0	0.13	0.07	0.11	0.18	0.18	0.38	0.23	0.51
IL-15	0.46	0.15	0.94	0.26	0.13	0.44	0.15	1.46	0.28	0.01
IL-17	0	0	0.33	0.18	0.11	0	0	1.81	0.54	0.01
Eotaxin	1.28	0.6	4.32	0.7	0.01	1.7	0.9	3.96	0.65	0.06
G-CSF	0.26	0.11	1.43	0.2	0.00	0.22	0.1	2.32	0.31	0.00
GM-CSF	0.58	0.34	2.01	0.73	0.10	0.42	0.37	1.68	0.82	0.19
IFN-γ	4.64	2.53	49.49	4.86	0.00	5.69	5.37	50.67	4.8	0.00
IP-10	10.2	3.12	6276.5	342.19	0.00	10.35	2.69	8551.2	914.25	0.00
MCP-1	375.77	20.23	2101.4	214.09	0.00	312.34	16.51	3251.9	808.87	0.01
MIP-1α	0.49	0.27	6.42	0.5	0.00	0.42	0.28	8.13	0.59	0.00
MIP-1β	0.58	0.19	214.15	6.51	0.00	0.31	0.18	254.97	8.62	0.00
RANTES	2.93	0.45	7013.9	1480.36	0.00	1.68	0.34	8772.5	1619.96	0.00
TNF-α	0.08	0.08	17.25	1.8	0.00	0.18	0.1	20.81	3.45	0.00
PDGFbb	1.04	0.4	2.67	0.54	0.03	0.87	0.28	3.78	0.76	0.01
FGFbasic	2.84	1.36	9.38	2.33	0.03	1.16	0.76	9.09	1.88	0.00
VEGF	238.97	13.36	382.15	21.41	0.00	278.85	29.37	393.38	26.17	0.01

Table 2. *Ex vivo* spleen effect of H2O extracts on Cytokine/Chemokine expression.

Uninfected mice - no virus in culture												
	H2O		*Eang*1		*Epur*1		*Eang*2		*Epur*2		*Epara*	
Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1α	226.3	28.8	*108.17	7.6	178.6	12.1	*128.26	16.6	154.5	22.9	*137.82	3.9
IL-1β	81.6	9.0	62.8	9.4	*29.82	5.2	*43.93	3.7	44.5	11.5	*35.38	5.2
IL-2	12.1	4.4	7.3	1.7	14.9	2.9	16.8	5.1	7.2	0.7	17.6	3.9
IL-4	2.1	0.3	1.4	0.3	*4.57	0.6	1.8	0.5	3.2	1.0	1.8	0.2
IL-5	14.5	1.8	9.9	2.0	*36.96	0.8	10.4	1.4	*25.02	0.4	*7.02	2.1
IL-6	1410.6	42.7	*533.36	27.4	*389.72	34.6	*698.01	120.3	1173.6	167.0	*660.56	154.9
IL-10	667.6	50.8	*1656.33	71.2	494.0	150.8	1152.6	350.7	939.0	332.4	621.6	160.5
IL-12p40	3.4	1.0	1.0	0.5	0.6	0.4	4.5	3.0	1.2	1.2	0.0	0.0
IL-12p70	0.8	0.8	0.0	0.0	0.0	0.0	0.8	0.8	0.0	0.0	0.0	0.0
IL-15	18.1	9.6	1.5	1.5	7.2	7.2	12.5	10.3	0.0	0.0	3.1	3.1
IL-17	13.1	6.6	30.5	11.9	*136.56	17.8	35.1	20.3	*324.54	74.8	36.4	21.3
IFN-γ	8531.8	1968.1	6878.1	871.2	*308.20	95.6	*1779.75	479.7	*131.76	57.2	1656.4	271.7
TNF-α	219.8	54.2	163.2	21.9	151.0	13.4	140.3	26.8	256.9	2.2	125.4	28.0
Eotaxin	0.6	0.6	0.2	0.2	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.0
IP-10	522.4	125.0	526.4	65.2	212.9	86.3	506.0	86.1	232.3	107.1	376.2	61.6
KC	1467.2	494.1	904.4	230.6	1320.7	345.7	1206.3	505.2	1690.6	190.1	1158.9	497.7
MIP-1α	1019.1	266.3	897.7	68.3	*1988.61	53.5	1215.4	180.3	*1961.10	176.4	1170.8	186.3
MIP-1β	969.6	312.3	1318.1	71.5	*2459.62	335.8	1639.0	290.3	2151.6	520.3	1164.3	63.9
MCP-1	76.6	9.2	49.4	14.5	113.0	25.8	79.0	31.7	81.9	7.4	77.3	31.0
Primed mice - no virus in culture												
	H2O		*Eang*1		*Epur*1		*Eang*2		*Epur*2		*Epara*	
Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1α	288.2	41.0	*103.38	5.1	187.4	47.4	*112.37	18.7	172.4	10.2	*115.07	27.9
IL-1β	55.0	7.0	48.7	8.7	*20.72	3.3	36.5	7.5	*31.26	4.9	*20.37	4.5
IL-2	5.0	0.6	5.2	0.6	*9.43	0.3	*10.90	0.3	5.6	0.7	*11.93	2.1
IL-4	1.1	0.2	1.0	0.3	*2.48	0.4	2.1	0.9	2.4	0.5	2.1	0.8
IL-5	6.4	3.4	5.5	0.9	13.2	3.2	5.6	1.1	14.6	0.7	0.0	0.0
IL-6	3077.7	539.0	*689.85	91.0	*289.16	104.7	*922.77	117.0	*1197.11	176.3	*906.07	134.5
IL-10	348.6	54.7	*1453.03	55.5	378.7	39.6	*984.02	77.0	*665.00	102.5	360.4	37.4
IL-12p40	1.2	0.6	1.1	1.0	0.0	0.0	2.0	0.7	0.0	0.0	0.2	0.2
IL-12p70	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.0	0.0	0.0	0.0
IL-15	0.0	0.0	0.0	0.0	0.0	0.0	12.1	8.8	0.0	0.0	0.0	0.0
IL-17	15.4	9.4	22.9	4.4	39.2	1.5	26.5	12.9	108.4	25.9	47.8	27.3

Table 2. continued.

Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IFN-γ	12109.6	322.0	*5666.29	1615.8	375.2	115.7	*3348.25	704.7	*148.02	1.7	*2388.17	502.2
TNF-α	243.2	26.5	*162.92	5.0	154.9	13.1	165.6	18.3	290.6	18.1	*125.15	15.2
Eotaxin	0.0	0.0	0.3	0.3	0.0	0.0	1.1	0.8	0.0	0.0	0.0	0.0
IP-10	413.1	35.7	401.3	47.8	*116.07	66.3	457.3	73.6	*134.91	24.7	292.5	43.4
KC	1701.9	265.8	*942.20	88.1	1266.8	214.1	1418.8	136.3	2081.6	237.6	1604.8	297.8
MIP-1α	1167.6	87.6	*581.05	28.6	*1886.95	150.1	1104.6	35.4	*2074.65	20.8	1098.3	155.2
MIP-1β	767.2	58.5	*977.59	23.8	2297.0	534.5	*1397.67	133.7	*1919.62	283.7	889.3	123.1
MCP-1	158.7	28.0	*51.96	7.2	102.0	29.2	85.1	20.1	86.2	22.0	117.6	16.8
Primed mice - stimulation with virus in culture												
	H2O		*Eang*1		*Epur*1		*Eang*2		*Epur*2		*Epara*	
Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1α	313.0	56.9	*144.61	23.9	166.0	18.6	*111.76	16.0	*137.70	16.9	145.9	5.2
IL-1β	61.1	6.1	49.4	7.5	*27.24	7.0	*30.70	5.7	*31.80	3.4	*24.43	6.5
IL-2	10.9	1.7	6.5	0.7	15.8	2.2	7.3	0.7	11.3	0.7	11.6	2.7
IL-4	2.4	0.7	1.7	0.3	8.9	3.0	2.8	0.7	4.0	1.1	4.8	2.0
IL-5	15.9	2.0	15.6	4.8	*65.65	15.1	25.9	6.0	43.5	16.7	22.2	5.9
IL-6	1882.0	103.9	*882.92	141.1	*546.37	89.8	*759.54	93.1	*1275.90	152.6	*991.19	29.6
IL-10	1272.5	236.5	1948.2	288.3	782.8	92.6	1291.4	175.7	966.9	51.6	1133.8	50.1
IL-12p40	0.6	0.5	0.3	0.2	0.0	0.0	0.0	0.0	0.6	0.3	1.2	0.9
IL-12p70	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-15	4.4	4.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	3.4
IL-17	21.7	4.8	31.6	8.4	45.0	9.8	23.9	2.4	*83.16	19.5	38.8	13.7
IFN-γ	12416.4	64.7	11420.2	529.7	*4628.22	833.3	10552.5	1001.4	*2497.28	385.4	10713.9	1647.9
TNF-α	203.6	40.4	182.6	30.9	157.7	15.6	128.9	20.6	269.1	49.9	148.6	19.6
Eotaxin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.5	0.3
IP-10	611.1	31.2	477.9	70.2	*308.55	52.3	497.3	47.2	*264.80	19.1	529.3	26.3
KC	1108.0	139.3	1036.9	111.4	1107.5	61.3	832.3	74.1	*2041.58	258.7	1459.1	227.8
MIP-1α	1009.8	119.6	688.7	101.0	*1896.17	65.6	873.8	56.5	*1830.45	83.9	1049.3	39.1
MIP-1β	893.8	68.2	1065.9	161.8	2837.4	674.4	1052.6	66.9	*1675.26	144.9	*1238.38	92.2
MCP-1	162.4	46.4	68.6	18.1	192.2	70.9	85.8	25.7	209.5	61.8	145.9	30.8

SEM – standard error of the mean. *, $p<0.05$, + $p<0.1$ for treatment group compared to vehicle control.

Table 3. *Ex vivo* spleen effect of EtOH extracts on Cytokine/Chemokine expression.

Uninfected mice - no virus in culture										
	EtOH		*Epur*1		*Eang2*		*Epur*2		*Epara*	
Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1α	232.61	47.78	*11.62	4.58	*73.38	29.16	*3.63	3.63	*19.64	12.31
IL-1β	117.27	18.39	*10.95	8.31	*24.97	16.12	*8.46	5.89	*15.68	8.13
IL-2	16.13	2.37	*0.00	0.00	*37.78	3.54	*0.00	0.00	+5.43	5.06
IL-4	1.85	0.74	4.28	3.85	2.01	0.50	2.72	0.11	+4.49	0.68
IL-5	27.56	5.84	*0.00	0.00	*7.09	1.12	*0.00	0.00	*2.86	2.86
IL-6	2127.21	523.09	+26.84	26.84	+286.29	67.33	+18.43	9.16	7+7.23	43.68
IL-7	2.26	1.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IL-9	0.00	0.00	14.84	13.13	*0.00	0.00	0.00	0.00	8.63	8.63
IL-10	856.15	171.01	*3.91	2.38	*309.39	100.93	*6.70	3.88	*27.71	20.24
IL-12p40	4.18	0.83	*0.00	0.00	*0.00	0.00	*0.00	0.00	*0.40	0.40
IL-12p70	14.19	1.77	*1.14	0.31	*1.88	1.88	*0.00	0.00	*0.71	0.71
IL-13	118.35	31.16	+7.58	7.58	+22.25	22.25	+0.00	0.00	+7.58	7.58
IL-15	31.49	3.70	*5.43	5.43	33.94	12.30	*0.00	0.00	*8.29	4.30
IL-17	18.16	5.67	+0.17	0.17	24.36	15.86	+0.00	0.00	+0.85	0.49
IFN-γ	12069.78	320.93	*0.00	0.00	*447.52	358.50	*0.00	0.00	*1.66	1.66
TNF-α	230.84	27.71	*2.78	2.78	*67.16	19.21	*0.70	0.70	*14.10	6.25
IP-10	711.78	115.60	*3.22	2.17	*241.81	119.13	*3.46	0.77	*11.76	4.32
KC	1298.53	479.98	+72.00	69.36	1961.13	496.20	+40.81	31.54	716.35	452.19
G-CSF	2155.14	871.88	+14.16	14.16	916.41	180.49	+15.27	11.70	218.21	151.61
GM-CSF	1161.24	128.49	*25.12	4.65	*167.27	86.96	*0.00	0.00	*22.09	5.36
MCP-1	129.25	54.55	+3.75	3.75	+430.68	141.44	+0.00	0.00	123.34	123.34
MIP-1α	1364.13	38.17	*13.90	10.73	1354.77	223.85	*43.39	8.71	*68.76	52.38
MIP-1β	1401.34	239.21	*24.73	13.79	1102.87	268.39	*23.28	7.69	*93.15	51.50
MIP-2	4954.18	1842.07	+322.75	235.41	4213.56	892.23	+510.70	92.94	+1326.91	768.02
RANTES	216.36	9.86	*8.66	1.68	*88.18	21.34	*32.16	3.47	*11.30	3.95
Primed mice - no virus in culture										
	EtOH		*Epur*1		*Eang2*		*Epur*2		*Epara*	
Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1α	527.47	112.97	*1.02	1.02	*21.32	12.41	*3.74	2.57	*4.25	4.25
IL-1β	127.02	5.71	*4.95	4.95	*22.79	6.28	*7.74	4.23	*13.60	7.91
IL-2	9.38	0.54	*0.00	0.00	*23.60	7.63	*0.00	0.00	*0.00	0.00
IL-4	1.18	0.23	1.28	0.89	1.25	0.28	2.00	1.38	4.51	1.91
IL-5	16.17	2.86	*0.00	0.00	*3.10	1.90	*0.00	0.00	*0.00	0.00
IL-6	4922.90	591.06	*3.13	2.64	*103.57	35.54	1*0.41	5.01	3*4.32	15.87
IL-7	4.06	1.33	+0.00	0.00	+0.00	0.00	+0.00	0.00	+0.00	0.00
IL-9	0.00	0.00	12.81	12.81	0.00	0.00	0.00	0.00	+35.68	17.37
IL-10	514.49	100.51	*1.37	1.37	*102.31	31.04	*3.65	2.23	*8.54	5.17

Table 3. continued.

Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-12p40	5.59	1.46	*0.00	0.00	*0.82	0.50	*0.00	0.00	*0.00	0.00
IL-12p70	24.75	1.58	*0.95	0.95	*0.58	0.33	*0.29	0.29	*0.44	0.28
IL-13	119.18	33.59	*11.37	6.56	*0.00	0.00	*0.00	0.00	*11.38	11.38
IL-15	40.06	8.03	*0.00	0.00	+18.18	6.99	*0.00	0.00	*6.91	3.93
IL-17	25.63	6.66	*0.00	0.00	*4.97	4.08	*0.00	0.00	*0.13	0.13
IFN-γ	13938.98	491.29	*0.00	0.00	*17.11	12.53	*0.00	0.00	*0.00	0.00
TNF-α	361.78	63.11	*1.18	1.18	*35.62	13.22	*0.52	0.52	*11.60	6.92
IP-10	663.11	43.27	*2.55	1.49	*38.40	16.22	*4.64	1.76	*5.92	2.36
KC	2114.40	324.37	*2.99	2.58	1603.25	600.95	*15.39	8.54	*263.61	189.74
G-CSF	3882.12	281.53	*0.00	0.00	*691.89	317.20	*6.87	4.27	*56.84	41.46
GM-CSF	1483.57	174.57	*4.18	4.18	*37.55	15.21	*4.18	4.18	*10.64	6.42
MCP-1	405.94	106.39	*0.00	0.00	732.41	287.93	*0.00	0.00	*11.90	11.90
MIP-1α	1588.62	161.24	*3.29	2.10	+847.21	337.46	*31.03	13.43	*41.07	29.85
MIP-1β	1099.67	57.63	*2.65	2.65	634.29	259.27	*16.80	8.11	*48.54	28.37
MIP-2	13276.35	4017.53	*71.62	15.48	+3365.99	1275.45	*306.40	130.91	*740.09	452.58
RANTES	234.82	23.80	*9.64	1.59	*48.71	8.50	*25.33	2.28	*8.28	2.49
Primed mice - stimulation with virus in culture										
	EtOH		*Epur*1		*Eang*2		*Epur*2		*Epara*	
Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1α	441.85	96.81	*7.54	6.57	*148.49	55.12	*8.03	2.60	*12.55	2.75
IL-1β	133.07	3.94	*7.74	5.05	*45.59	7.70	*9.28	4.28	*8.44	5.19
IL-2	22.19	0.53	*0.28	0.28	*69.01	12.65	+7.01	7.01	*2.81	1.30
IL-4	3.42	0.78	7.32	2.62	*10.44	1.93	4.41	1.81	7.02	2.37
IL-5	41.43	6.65	*0.00	0.00	*23.39	3.67	*0.66	0.66	*0.00	0.00
IL-6	2522.00	545.46	*29.77	14.77	+978.72	432.52	*34.20	17.77	*65.33	23.17
IL-7	1.70	0.55	*0.00	0.00	0.86	0.86	*0.00	0.00	*0.00	0.00
IL-9	0.00	0.00	+33.81	18.47	3.09	3.09	0.00	0.00	+20.16	10.16
IL-10	1343.54	303.95	*4.42	2.19	964.84	108.89	*23.30	19.41	*15.41	7.04
IL-12p40	3.67	1.01	*0.00	0.00	*0.82	0.38	*0.00	0.00	*0.00	0.00
IL-12p70	16.98	2.50	*0.66	0.43	*7.80	2.01	*0.51	0.51	*0.00	0.00
IL-13	145.45	24.49	*19.95	14.93	+254.38	47.17	*4.55	4.55	*9.10	9.10
IL-15	27.82	3.68	*2.42	1.48	32.91	6.78	*8.63	5.40	*4.18	2.56
IL-17	43.60	14.33	*0.08	0.08	+17.34	6.15	*0.44	0.44	*0.18	0.11
IFN-γ	14016.33	406.58	*0.00	0.00	*5262.26	1778.16	*3.21	3.21	*0.89	0.46
TNF-α	211.53	47.66	*4.33	3.00	+108.20	28.46	*5.01	4.19	*13.58	6.12
IP-10	731.54	57.76	2.93	2.93	*505.88	62.53	*8.98	6.31	*7.95	2.84
KC	1659.48	272.00	*68.53	57.48	+3120.73	822.51	*204.46	189.79	*316.14	127.85
G-CSF	2808.01	247.00	*16.27	14.82	2707.40	932.06	*77.81	70.51	*67.90	26.77
GM-CSF	1676.53	214.04	*19.60	6.35	*495.00	52.11	*14.24	6.37	*3.35	3.35
MCP-1	295.19	101.56	*1.90	1.90	*1191.08	224.36	+77.85	77.85	*18.80	9.03
MIP-1α	936.08	229.93	*17.58	13.19	*1859.98	135.32	*111.17	81.63	*60.10	28.20

Table 3. continued.

Cytokine	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
MIP-1β	1006.72	190.93	*27.74	20.87	*1796.58	167.45	*70.69	51.50	*76.07	27.51
MIP-2	5814.77	705.03	*313.11	213.48	7998.15	3401.07	*666.06	415.43	*806.57	332.08
RANTES	149.56	14.81	*10.16	2.92	*89.92	10.39	*24.15	2.95	*10.06	3.49

SEM – standard error of the mean. *, $p<0.05$, + $p<0.1$ for treatment group compared to vehicle control

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CHAPTER III. EFFECTS OF ALTERNATING WATER AND ETHANOL ECHINACEA SPP. EXTRACTS DURING DIFFERENT PHASES OF INFLUENZA INFECTION IN MICE

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Abstract:

Background: In influenza infection, the balance between activation of inflammatory and antiviral pathways and the appropriate down-regulation of immune response as virus is cleared is important in optimal recovery from infection. Early in infection, a delayed or compromised immune response to the virus can result in longer duration of infection or greater severity of infection. In the later days of infection, an overactive immune response to the virus can cause damage to the lung tissue. *Echinacea* is a dietary

supplement used to treat upper respiratory infections. Data suggests that *Echinacea* preparation in water results in immune-stimulatory effects, whereas ethanol preparations may result in anti-inflammatory effects. It was hypothesized that an "immune-stimulatory" water extract preparation of *Echinacea* during the early phase of influenza infection may promote appropriate antiviral immune activation, and that the administration of an ethanol extract preparation during the later phase of infection may minimize immunopathology.

Methods: In the first set of experiments, BALB/c mice were gavaged with *E. angustifolia* water extract (*Eang* H2O) days 1-5 of influenza virus infection, followed by daily gavage with *E. angustifolia* ethanol extract (*Eang* EtOH) until day 9 post-infection (p.i.). Inflammation was assessed using cytokine/chemokine analysis of the bronchoalveolar lavage fluid (BAL) and serum anti-influenza antibody was measured. In a second set of experiments, five different combinations of *Echinacea* extracts were used to identify optimal treatment strategies. Mice were gavaged with either *Eang* H2O water extract or a water vehicle (H2O) until day 5 p.i., followed by treatment with either *Eang* EtOH ethanol extract, *E. paradoxa var. paradoxa* ethanol extract (*Epara* EtOH), or EtOH vehicle control on day 6 until day 8 p.i.: 1) H2O/EtOH, 2) *Eang* H2O/EtOH, 3) *Eang* H2O/*Eang* EtOH, 4) *Eang* H2O/*Eang* EtOH, and 5) *Eang* H2O/*Epara* EtOH). The pattern of lung gene expression was analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID).

Results: In the first set of experiments, there was a trend towards a decrease in the viral titer of the *Eang* H2O/*Eang* EtOH treatment group in the BAL and lung. In BAL fluid, the pro-inflammatory cytokines IFN-γ, IL-6, TNF-α, and multiple chemokines (eotaxin,

MCP-1, RANTES, LIX, M-CSF, MIP-2) were significantly decreased ($p<0.05$) in the *Eang* H2O/*Eang* -treated mice compared to the H2O/EtOH control mice. Serum anti-influenza IgG was also increased in mice receiving *Eang* H2O/*Eang* EtOH treatment.

In the second set of experiments, five different treatment groups were used to compare different combinations of extracts on illness severity and gene expression with a control treatment group. No significant change in lesion scores or weight loss was observed in any of the treatment groups. However, the gene expression results demonstrated that the *Eang* H2O/EtOH vehicle treatment group up regulated a greater number of genes associated with lymphocyte activation and differentiation, whereas the *Eang* H2O/*Eang* EtOH and *Eang* H2O/*Epara* EtOH groups down-regulated expression of IL-8-like chemokines and several genes involved in wound healing.

Conclusions: Although the *Echinacea* treatments did not impact illness severity or lung lesion score, a reduction of inflammatory BAL cytokines and chemokines and enhanced anti-influenza serum antibody was observed, suggesting that the combination of *Eang* H20 followed by the *Eang* EtOH may have beneficial immunomodulatory effects that could be further optimized. Gene expression revealed different profiles for each combination analyzed also suggesting that the combination selected could impact the anti-inflammatory effects in the lung, an effect that could potentially reduce immunopathology if assessed at a subsequent time point.

Introduction:

Influenza is a seasonal infection that impacts the millions of people worldwide every year [1]. The ability of this virus to recombine with other strains adds to the high

variability in the strains present in circulation in a process termed antigenic shift. In addition, influenza is an RNA virus that has no proofreading mechanism to correct transcription errors. This adds to the mutations and antigenic drift seen in influenza seasons. Because mutations and re-combinations can lead to new viral strains of influenza, there is a high rate of treatment resistant strains of influenza. Currently, the seasonal vaccination for influenza is the best strategy for influenza treatment [2,3]. In some years, the match between influenza strains in the vaccine is not an optimal match with the strains of the virus that are circulating in the population. Anti-viral medications are effective in treating influenza; however, several anti-viral medications have already been discontinued due to emergence of resistant strains [2,4,5]. Ion channel targeted therapies, such as amantadine and rimantadine, are currently no longer effective against circulating strains of influenza. [6,7]. Also, influenza viruses have developed resistance to neuraminidase, a viral structure protein, inhibitors (oseltamivir) [4]. To combat the influenza resistance to these antivirals, one proposed treatment has been to use all three antivirals in combination. Although this approach has shown increased antiviral activity compared to each antiviral alone, it is not appropriate for all age groups and physical condition of the patients that would benefit the most from antiviral therapy, such as the older and immunosuppressed populations [8,9].

With decreased anti-viral treatments available and a trend towards more alternative medicine approaches, *Echinacea* has become a popular nutritional supplement in the U.S. *Echinacea* is a flowering plant that is native to North America, commonly harvested for its therapeutic value. There are nine known species of *Echinacea* [10]. Three of these species are used medicinally: *E. purpurea, E. angustifolia,* and *E. pallida*.

Echinacea can be used as a whole plant, aerial parts, or the roots. The plant part can be then combined with either water or ethanol (tincture). The solution the plant is prepared in dictates the active components present for immune activity due to solubility of the compounds. Water preparations may contain polysaccharides (hydrophilic compounds) and caffeic acid derivatives. Ethanolic preparations consist largely of alkamides (lipophilic compounds) and caffeic acid derivatives. *Echinacea* extracts have been shown to enhance the immune system, promote wound healing, and may have some anti-viral properties when administered as a water extract. In addition, some findings suggest that *Echinacea* extracts promotes an anti-inflammatory response when administered as an ethanol extract. Polysaccharides isolated from *Echinacea* preparations have been shown to be immunostimulatory, enhancing innate immunity and cellular adaptive immune responses, whereas alkamides have an anti-inflammatory effect decreasing inflammatory cytokines [11-15]. With respect to plant species, extracts prepared from *E. angustifolia* or *E. purpurea* are more widely used and have been evaluated in other studies. Although the *E. paradoxa var. paradoxa* species has not been traditionally used for medicinal purposes, one recent study showed that ketones isolated from *E. paradoxa var. paradoxa* extracts had anti-inflammatory effects on macrophages [16,17]. *E. paradoxa* ethanol extract has also been shown to inhibit the nitric oxide production in macrophages stimulated with LPS *in vitro* [17]. Therefore, although the potential effects of extracts prepared from *E. paradoxa var. paradoxa* species in influenza virus infection remain to be determined, findings from other studies demonstrating immunomodulatory effect support the possibility that this species may also have some benefits.

In influenza, an appropriate balance between the development of a potent immune response to clear the virus, and yet limit tissue damage is necessary for optimal recovery from the infection. An insufficient cellular and cytokine response lead to an insufficient immune response that results in prolonged recovery time and diminished challenge response; whereas, an overactive immune response adds to the immunopathology associated with fatal infections through cellular infiltration, inflammation, and fibrosis [18,19]. Increased cellular infiltration and hypercytokinemia (in highly pathogenic strains) are thought to contribute to lung injury during influenza infection [18]. Given that *Echinacea* appears to have different immunomodulatory properties, it may be possible to take advantage of the immunostimulatory properties during the first few days of viral infection by administering water extracts, and follow this with ethanol-based extracts that may limit inflammation associated tissue damage. Although extracts derived from *E. paradoxa* have never been tested in an *in vivo* influenza infection model, previous findings have shown *E. paradoxa* ethanol extract reduced production of inflammatory factors including nitric oxide, and it is possible that extracts derived from this species may also have some benefit, as decreased nitric oxide production may limit acute lung injury [20]. To address these balance issues with influenza, we propose to utilize a combination of *Echinacea* extracts administered at different time points during the infection.

Echinacea has been shown to be immunostimulatory when prepared as a water extract and anti-inflammatory when prepared in as an ethanolic extract. According to the hypotheses proposed for these studies, it was predicted that administration of *Echinacea* water extracts during the first several days of infection would results in enhanced innate

immune activation including dendritic cell priming and T helper activation culminating in increased antibody response. It was also hypothesized that administration of ethanol extracts during the later phase of infection would limit inflammation-associated tissue damage by decreasing pro-inflammatory cytokines. Finally, it was expected that extract administration would potentiate gene expression of genes important in stimulating adaptive immunity, and would decrease inflammatory-associated gene expression. The *Echinacea* combination treatment is a novel approach to modulating the immune response to influenza infection.

Material and Methods:

Overall experiment design

Two sets of experiments were performed. In the first experiment, the commonly used *E. angustifolia* extract was evaluated. Mice were gavaged with either an *E. angustifolia* water extract or a water vehicle until day 5 post-infection (p.i.), and then with *E. angustifolia* ethanol extract or ethanol vehicle until termination of the experiment at day 9 p.i. This design allowed us to test whether the combination treatment would provide immunostimulatory effects resulting in enhanced T or B cell immunity, and also limit the inflammatory-associated tissue damage. In a second experiment, the same *E. angustifolia* treatment was tested, but this time was compared with extracts obtained from *E. paradoxa* given the recent finding that this species may also have anti-inflammatory effects. Again in these experiments, water extract or vehicle was administered until day 5 post-infection, followed by ethanol extract or vehicle from day 6 until the termination of the experiment at day 8 p.i. The purpose of the first experiment was to assess

"traditional" immune measures: weight loss, viral titer, lung cell populations, and cytokine/chemokine expression. The second experiment was designed to determine the effect of different combinations of *Echinacea* species and extracts on basic illness measures and gene expression in response to influenza infection.

Mice.

Male BALB/c mice (2 months of age) were purchased from Charles River laboratory and allowed to acclimate for several weeks prior to the start of the experiment (Experiment 1, n=27; Experiment 2, n=90). Iowa State University Committee on Animal Care approval was obtained for all procedures that were utilized in the care and experiments involved with the mice. The mice were housed separately to accurately evaluate illness severity measures.

Plant and extracts.

Echinacea angustifolia (Eang) (accessions PI631285, A28187) and *Echinacea paradoxa var. paradoxa* (accession PI633663) plants were grown and extracts at Iowa State University. *E. angustifolia* (PI631285, A28187) and *E. paradoxa* (PI633663) were harvested in USDA North central regional plant introduction station at Ames, IA (USA). Water extracts were prepared by boiling 6 grams of dried root in 100 milliliters (ml) of endotoxin free water in an endotoxin free flask, and stirred for 1 hour at room temperature. Extracts were filtered using endotoxin free glass filter paper, centrifuged for 15 minutes at 10,000 rpm and pellets discarded before freeze drying. The ethanol extracts were prepared from the dried ground root powder which was placed into a Whatman 43 × 123-mm cellulose extraction thimble (Whatman International Ltd.,

Maidstone, UK) with glass wool. The roots were refluxed with 250 ml of ethanol as an organic solvent for 6 hours using a Soxhlet extraction device [12]. The extract was dried using an R-114 rotary evaporator (Brinkman Instruments, Westbury, NY) at <30°C under reduced pressure and re-suspended in 43% ethanol. These extracts were stored in concentration solution at -20°C until aliquots were made for testing. Water and ethanol extracts were diluted to 14.67 mg/ml of extract for the experiments.

Gavage treatment and infection.

Animal feeding needles (20 gauge x 1-1/2, Cadence Science, Staunton, VA) were used to gavage the mice. In the first experiment, there were two treatment groups, 1) *E. angustifolia (Eang*) H2O extract+ *Eang* EtOH extract, and 2) H2O vehicle+EtOH vehicle. In this first experiment, mice were gavaged either with *E. angustifolia* (*Eang*) water or vehicle (H2O) solutions starting one day prior to infection and for the first 5 days following infection. This treatment was then followed by gavage with either *Eang* EtOH extract or EtOH vehicle for days 6-9 of infection. Mice were euthanized day 9 post-infection. *Echinacea* extracts were administered at a concentration of 110 mg/kg. In the second experiment, there were 6 treatment groups: 1) H2O/EtOH; 2) *Eang* H2O/EtOH; 3) *Eang* H2O/*Eang* EtOH; 4) *Eang* H2O/*Epara* EtOH; 5) H2O/*Eang* EtOH; 6) H2O/*Epara* EtOH. Similar to experiment one, mice were gavaged with *Eang* water extract or H2O vehicle one day before infection and during the first 5 days of infection. Then, on days 6-8 of infection, mice were gavaged with one of the following solutions: *Eang* EtOH, *E. paradoxa var. paradoxa* (*Epara*) ethanol extract, or EtOH only control. In experiment two, all mice were euthanized day 8 post-infection (p.i.). In both experiment one and experiment two, mice were infected with 30µl of influenza virus,

A/PR/8/34, H1N1 (HA=8192, at $10^{10.45}$ EID50/ml) via an intranasal route given under isoflurane anesthesia.

Disease severity and viral load.

Body weight was measured daily as a measure of illness severity post-infection. Viral titer in lungs and BAL fluid from mice in the first experiment was assessed by the Iowa State University Veterinary Diagnostic Laboratory using primers to detect influenza virus by fluorogenic reverse transcription polymerase chain reaction (real-time reverse transcription PCR) in the lung tissue samples. The apical, azygous, cardiac, and diaphragmatic lobes were submitted for viral titers from the first experiment only. Primers specific to the virus conserved region of the SIV NP gene were used with TaqMan® chemistry. Sequences from GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html) and Influenza Sequence database (www.flu.lanl.gov) were utilized. A commercial vendor synthesized the forward primer (SIVRTF: 5'-CGGACGAAAAGGCAACGA-3'), reverse primer (SIVRTR: 5'-CTGCATTGTCTCCGAAGAAATAAG-3') and a TaqMan® MGB reporter probe FAM (6-carboxyfluorescein) and a non-fluorescent quencher (SIVRTP: 5'-6FAM-CCGATCGTGCCYTC). Primers were synthesized by Integrated DNA Technologies (Coralville, IA), and the probes were synthesized by Applied Biosystems (Foster City, CA). Viral RNA was extracted from 50µl of lung tissue using the Ambion® MagMAX™ Viral RNA Isolation kit (Applied Biosystems) and a KingFisher® 96 magnetic particle processor (Thermo Scientific, Waltham, MA). H1N1 and H3N2 swine influenza viruses and elution buffer were used as positive and negative controls, respectively. The QuantiTect® Probe RT-PCR Kit by Qiagen (Valencia, CA) was used

to perform the real time reverse transcription-PCR. Each reaction consists of 4µl of lung sample with 0.4µM (final concentration) of each primer and 0.2µM (final concentration) of the probe for a total volume of 20µl for each reaction. Using a 384 well format and the ABI 7900HT Sequence Detection System (Applied Biosystems), reverse transcription occurred for 30 minutes at 50°C. Immediately following reverse transcription, the RT-PCR was activated with 15 minutes at 95°C. Activation was followed by 40 cycles of 15 seconds at 94°C and 60 seconds at 60°C. To generate a standard curve, a set of influenza preparations with known concentrations of viral titers (EID50/ml) were used. Each sample then could be extrapolated by using the standard curve to convert the threshold cycle (Ct) value to a viral titer [21].

Lung pathology.

Lung tissue from left cranial lobe from mice in the second set of experiments was submitted to the ISU Veterinary Diagnostic Laboratory for lesion scoring. Lungs were immersion-fixed in 10% buffered formalin, processed, sectioned, and stained with hematoxylin and eosin for histopathological examination. The remaining lung lobes were utilized for RNA extraction. The tissues were embedded in paraffin wax and sectioned at 5-µm thickness. These sections were then stained with hematoxylin and eosin (H&E), and examined by light microscopy. Lungs were examined for bronchiolar epithelial changes. These changes included attenuation, proliferation, degeneration, and necrosis. Severity of peribronchiolar and alveolar inflammation were also evaluated. Lungs were scored from 0 to 3 to reflect an estimate of the percentage of lung tissue containing lesion and the severity of lesions. The lung sections were scored according to the following criteria: 0, no significant lesions or minimal epithelial cells change in <25% of the lung

tissue; 1, mild to moderate epithelial cell changes and interstitial pneumonia in ~25%–50% of the tissue; 2, moderate epithelial cell changes and moderate interstitial pneumonia in ~50%–75% of the tissue; and 3, significant epithelial cell changes and moderate to severe interstitial to bronchointerstitial pneumonia in ~75%–100% of the tissue [21]. One pathologist performed the scoring and remained blinded to the treatment groups during analysis.

qRT-PCR.

The lung (for experiment 1 the left lobe and in experiment two the remainder of the lung) was homogenized with a glass grinder, and RNA was extracted using a TRIzol® extraction method utilizing 1 ml of TRIzol® per 100 mg of lung tissue. Grinders were washed with 1 ml of TRIzol® after each lung. Samples were kept on ice until all lungs were processed and allowed to come to room temperature before proceeding to phase separation. Phase separation was conducted using 200 µl of chloroform per 1 ml of TRIzol®. Samples were vortexed for at least 30 seconds and centrifuged for 15 minutes at 4°C at 12,000 x g. The colorless aqueous phase was removed and used for RNA isolation. One ml of the aqueous phase was combined with 400 µl of COLD isopropanol in a 1.5 ml Eppendorf tube. After vortexing, the samples were centrifuged at room temp for 15 minutes at 12,000 x g. The RNA pellet was washed using 200 µl of 75% ethanol and centrifuged at room temp for 15 minutes. Ethanol was removed without disrupting the pellet and allowed to air dry. Pellets were re-suspended in 100 µl of RNAse free water. Using the Qiagen® RNeasy® Mini prep kit, following the protocol provided by the company for animal tissues, and the on-column DNase digestion optional step, the RNA was prepared and then converted to complimentary DNA (cDNA) using the RT2

first strand kit by Qiagen®. The cDNA was then analyzed in the Qiagen® qRT-PCR microarray for inflammatory cytokines/chemokines and T and B cell activation pathways. The T and B cell activation array kit was used for the first set of experiments and included mice from the following treatment groups: 1) H2O/EtOH (n=3), and 2) *Eang* H2O*/Eang* EtOH (n=3). In the second set of experiments, both the T and B cell activation array and the Inflammatory cytokine and chemokine array kits were used with the following treatment groups: 1) H2O/EtOH (n= 6), 2) *Eang H2O/*EtOH(n=5), 3) *Eang* H2O*/Eang* EtOH (n= 7), 4) *Eang H2O/Epara* EtOH (n=6) , 5) uninfected (n=3). The H2O/*Eang* EtOH and H2O/*Epara* EtOH groups were not analyzed for gene expression.

Flow cytometry.

BAL cells were harvested by centrifugation at 1200 revolutions per minute (rpm) for 10 minutes and re-suspended in 250 µl of BD stain buffer (PBS- azide+0.1%BSA, BD Biosciences). Samples were treated with 2 mls of ice cold 0.015M ammonium chloride red blood cell lysing agent for 10 minutes, washed in BSA and re-suspended in 250 µl BSA. Cell counts were obtained using the Z™ series Coulter Counter® (Beckman-Coulter, Inc., Brea, CA). Cell count for each sample was determined and adjusted to a range from $1x10^5$ and $1x10^6$ per ml. Samples less than $1x10^5$ cells per ml were combined with another sample from the same treatment group. For flow cytometry, each sample was treated with 10 µl of Fc block 1:10 dilution (0.5mg/ml, BD Biosciences) for 10 minutes. Cells were stained with PE anti-mouse GR-1, FITC anti-mouse CD11b, and PE-Cy7 anti-mouse CD45. Cell populations were defined as follows: neutrophils (PMNs) defined as CD45+CD11b+Gr1+, high side scatter SSC+; inflammatory

monocytes (i-Monos) defined as CD45+, CD11b+, Gr1+; constitutive monocytes (cMonos) defined as CD45+, CD11b+, Gr1-, low side scatter; and lymphocytes defined as CD45+, CD11b-, Gr1-, low side scatter). Samples were mixed and incubated for 30 minutes at 4°C protected from light. After incubation, 1 ml of BD stain buffer was added to each sample. The samples were centrifuged at 1200 rpm for 5 minutes at 2-4°C. Supernatant was removed by vacuum suction and 2 more wash steps were performed using 1 ml of BD stain buffer for each wash. After the final wash step, supernatants were removed, cells were gently mixed by placing the plate on a vortex, and 150 µl of diluted BD™ stabilizing fixative (BD Biosciences) was added to each sample. Samples were then transferred to 5 ml BD Falcon™ tubes and stored at 4°C until analyzed on a BD FACSCanto™ Flow Cytometer (BD, Franklin Lakes, New Jersey) by Iowa State University Flow Cytometry Facility.

Multiplex Assay.

Bronchoalveolar lavage fluid (BAL) was collected to measure cytokine/chemokine concentration. The BAL was separated into supernatant and cellular portions by centrifugation at 1200 rpm for 10 minutes. The Milliplex® microsphere (EMD Millipore Corporation, Billerica, MA) assay was used to analyze BAL supernatant for expression of different cytokines and chemokines that could be present in the lung of the infected mice. The preparation of the plate was performed according to manufacturer's instructions. The plate was read using Bio-plex® Suspension Array System (Bio-Rad laboratories, Inc., Hercules, CA) instrumentation. Data was exported from Bio-plex® Manager to Microsoft® Excel (Microsoft, Redmond, WA) for further analysis.

Serum antibody.

Blood was collected by cardiac puncture and centrifuged at 600 x g for 15 minutes. Sera were stored at -80 °C until tested by indirect ELISA for anti-influenza IgG (H&L). ELISA plates (Immulon, Alexandria, VA) were coated with influenza virus A/PR/8/34 diluted in carbonate coating buffer (pH = 9.6) at a concentration of 200 HAU/ml for anti-influenza-IgG (H&L) and incubated overnight at 4 °C. After blocking with 0.1% BSA in saline solution at 37°C for one hour, plates were washed three times with PBS/0.05% Tween 20. Three washes with PBS/0.05% Tween 20 were utilized between each step. Samples were tested in duplicate. After 3 hours of incubation at 37°C, alkaline phosphatase-conjugated goat anti-mouse IgG (H&L) was added. After overnight incubation at 4°C, phosphatase substrate (Sigma-Aldrich, St. Louis, MO) was added and incubated at room temperature for 20 minutes. Optical density (OD) of each well was measured using a microtitration plate reader (BMG FLUOstar Galaxy) at a 405 nm wavelength.

Analysis.

Statistical analysis was performed with an analysis of variance (ANOVA) with post hoc least significant difference (LSD), and independent student t-test using SPSS® software version 20 (IBM Corporation, Armonk, New York). For body weight analysis, a repeated measures ANOVA was used. The independent student t-test was used to evaluate the outcome measures: BAL cytokines and chemokines, BAL immune cell subpopulations, as well as viral load in the lungs. SABiosciences™ microarrays were analyzed: T and B cell activation array, House-keeping gene (HSK) Hsp90ab1,

Inflammatory Cytokines and Chemokines, HKG-Hsp90ab1. Results were obtained using SABiosciences™ Web-based analysis software, RT^2 Profiler PCR Array Data Analysis version 3.5**.** Housekeeping gene selection was based on the automatic selection in the Web-based software analysis. This gene was selected because it was consistently <1 Ct change between all the groups analyzed. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was utilized for data-mining and functional annotation and analysis of SABiosciences™ Array data. Extracts were evaluated for gene fold changes compared to uninfected mice and then again to mice treated with vehicle control in separate analysis. The fold change data exported from SABiosciences Web based software analysis was imported into DAVID and analyzed against a murine background with classification stringency (default setting). Statistical threshold for selection of fold changes was x>1.5 for up regulation and <0.667 for down regulation. Enrichment score for each cluster reported was selected as > 1.3 which is equal to a geometric mean of all the enrichment p-values <0.05 in the cluster (EASE score). EASE score (p-value) is used to evaluate the significant of a gene-term enrichment for each gene annotation. For a gene functional classification cluster, the enrichment score is a geometric mean of all the EASE scores in the cluster. Enrichment score for each cluster was selected as > 1.3 which is equal to a geometric mean of all the enrichment p-values and represents a p-value<0.05. Bonefferoni values corrected for multiple testing effects which is a conservative approach to data mining which is utilized to prevent false discovery rates in large gene backgrounds that return lower EASE scores and can result in false interpretation of the data. In this study, a Bonferroni value of <0.10 was considered significant because of the size of the gene input list. This study submitted a

pool of roughly 170 genes. DAVID gene input lists range from 1-10 genes to much larger gene lists and whole genomic sequences [22].

Results:

Daily body weight was used to assess illness severity, and in the first experiment, there were no significant differences in weight loss between the H2O/EtOH control treatment and *Eang* H2O/*Eang* EtOH extract treatment (Figure 1), although the daily weight loss did differ from uninfected mice as expected (significant interaction, $p < 0.05$). In the second experiment, the six treatment groups including a vehicle control were compared to an uninfected control, (Figure 2). With respect to Experiment two, the results from a repeated-measures-ANOVA showed a significant treatment by time interaction such that the daily weight loss for the infection period was not the same across groups ($p< 0.05$). However, in follow-up post hoc analyses, only weight loss in the uninfected mice was significantly different than all other groups (as expected) with no significant differences observed between treatments. However, there were no differences in the daily weight loss between any of the *Echinacea* treatment groups and vehicle treatment suggesting that *Echinacea* treatment did not alter the severity of infection as measured by weight loss during the eight day infection period. If weight loss was examined only during the early phase combining the same treatment groups together (all *Eang* treated mice compared to all vehicle treated mice), no significant effects of extract were found.

In experiment 1, viral titer was evaluated as another measure of illness severity. Although at day 9 p.i., viral clearance has already begun and may not reflect peak viral

loads observed earlier during the infection. All infected mice had detectable virus in the lungs and BAL at this time point. Mice treated with *Eang* H2O/*Eang* EtOH showed a trend towards reduced viral load present in the BAL fluid (p=0.08) compared to the mice treated with the vehicle control. The lung titer results were in the same direction as the BAL results, with a trend towards a decrease in viral titer with mice that received the *Echinacea* combination (p=0.08) (Figure 3).

In the second set of experiments, lung tissue was used to examine the extent of histopathology and to examine gene expression, and therefore tissue was not available to assess viral load. In this second set of experiments, lesion scores were determined by the size and extent of the pathology (Figure 4a). A lung index was determined by multiplying the lesion score by the percent of the lung involved (Figure 4b). No significant differences in lesion scoring or lung index were observed between any of the *Echinacea* treated groups compared to the mice treated with vehicle control (H2O/EtOH).

The BAL cell populations were compared between *Eang H2O/Eang EtOH* treated mice and H2O/EtOH on day 9 p.i. (experiment 1). The total number of cells was not significantly different between groups (Figure 5a). Cell populations were analyzed for overall cell count (Figure 5b) and percent of total cell count (Figure 5c). The total number of each cell population and the percentage of each population were not different between the *Eang* H2O*/Eang* EtOH treated mice and the H2O/EtOH treated mice, suggesting that at the time point measured, *Echinacea* combination treatment of the mice did not affect cell infiltration. It is possible that *Eang* H2O*/Eang* EtOH treatment alters cellular populations at an early phase of treatment and/or alters a population that was not isolated in this study.

To further analyze the impact of *Eang* H2O/*Eang* EtOH treatment, cytokine and chemokine concentration in the BAL fluid was analyzed from mice euthanized on day 9 p.i. (experiment 1) (Figure 6a-f). To establish the effect of infection on the concentration of cytokines and chemokines, infected mice from either treatment group are compared with uninfected mice in Table 1. It is apparent that infection results in significant increases in multiple cytokines and chemokines. To evaluate the effect of the extract combination on the cytokine/chemokine levels present in the lung at day 9, the *Eang* H2O/*Eang* EtOH treatment group was compared with the vehicle control. The cytokines IFN-γ, IL-6, and TNF-α were significantly decreased ($p<0.05$), with a trend towards a decrease in leukemia inhibitory factor (LIF) ($p=0.06$) and IL-12p70 ($p=0.10$) in the BAL of treated mice compared to the BAL of H2O/EtOH control mice. In addition, the concentration of multiple chemokines was significantly decreased ($p<0.05$) in *Eang* H2O/*Eang* EtOH treated mice including eotaxin, MCP-1, RANTES, LIX, M-CSF, and MIP-2. VEGF, a protein involved in angiogenesis and cell migration, was also decreased ($p<0.05$) in the BAL of mice treated with the *Eang* H2O/*Eang* EtOH combination.

To determine the effect of *Eang* H2O/*Eang* EtOH on the adaptive immunity, serum from the mice was analyzed for anti-influenza specific IgG levels. *Eang* H2O/*Eang* EtOH treated mice demonstrated a trend towards greater anti-influenza specific IgG level (Figure 7) compared to the H2O/EtOH treated mice ($p=0.06$).

To further analyze the effects of the *Echinacea* combination treatments on the immune response to influenza, lung gene expression data was evaluated using SuperArray Biosciences analysis. Genes associated with cytokines, chemokines, and T and B cell activation were both up-regulated and down regulated with infection at day 8.

Relative to vehicle control, the *Echinacea* combination in general increased genes related to T and B cell activation and decreased the cytokine/chemokine gene fold regulation (Figures 8 and 9).

To further understand the gene regulation in the lungs of mice treated with *Echinacea* combinations, the data-mining tool DAVID was used to determine the effect of treatment with *Echinacea* combination treatments compared to the H2O/EtOH control (Appendix). DAVID imports functional data from a variety of databases. The gene ontology (GO) was reported for the cluster unless otherwise specified in the cluster. Genes lists for up regulated and down regulated genes relative to infection are shown in Table 2 and 3; gene lists relative to vehicle control are shown in Table 4 and Table 5.

For experiment 1 (day 9 p.i), DAVID analysis returned only 2 genes with regulation changes in the *Eang* H2O/*Eang* EtOH group compared to H2O/EtOH control (Table 3 and Table 5). Gene clustering is not performed in groups less than 6. However, the Ccr8 (Chemokine (C-C) receptor 8 gene) was up regulated ($p<0.05$) and the CD3g (CD3 antigen, gamma polypeptide gene) was down regulated ($p<0.05$).

In experiment 2 (day 8 p.i.), the gene regulation fold change results varied by species of *Echinacea* used for treatment. Interestingly, the *Eang* H2O/EtOH treatment altered expression of more genes than the other *Echinacea* combinations (Figure 10 and 11). DAVID analysis returned 25 gene that were up regulated (Figure 10b and Table 3) and 7 genes that were down regulated with *Eang* H2O/EtOH (Figure 11b and Table 5) treatment compared to H2O/EtOH controls. For *Eang* H2O/EtOH up regulation, DAVID returned 10 annotations clusters with enrichment scores >1.3. Gene ontology (GO term)

evaluation suggested that *Eang* H2O/EtOH treatment enhanced cellular proliferation, differentiation of T cells, activation of the T cell receptor (TCR) through molecules Lck and Fyn, cell to cell communication and expression of adhesion molecules through external cell stimulation and enhanced phosphorylation.

For *Eang* H2O/EtOH down regulation, DAVID analysis returned 4 gene annotation clusters >1.3 (Appendix). Genes that were down regulated with fold enrichment were CRP (C-reactive protein), CCL4 (MIP-1β), CCL9 (chemokine ligand 9), CCL19 (MIP-3β), CCL20 (MIP-3a), TNF (tumor necrosis factor), and IL1f8 (IL-1 family 8). GO terms data suggested that *Eang* H2O/EtOH down regulated the cytokine/chemokine activity, signaling, and IL-8-like chemokines, and decreased the inflammatory response to damage or infection.

When *Eang* H2O treatment was instead followed by *Eang* EtOH treatment, a different set of genes were altered. *Eang* H2O/*Eang* EtOH treatment up regulated 3 genes compared to H2O/EtOH controls (Figure 9b and Table 4): PRLR (Prolactin receptor), IL11 (Interleukin 11), and Impdh1 (nosine 5'-phosphate dehydrogenase 1). Six genes were down regulated with *Eang* H2O/*Eang* EtOH compared to H2O/EtOH controls (Figure 9b and Table 5): CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL8 (MCP-2), TNF (tumor necrosis factor), and Tnfsf14 (tumor necrosis factor superfamily member 14). DAVID analysis returned 2 annotation clusters >1.3 (Appendix). Evaluation of the GO term data suggest that *Eang* H2O/*Eang* EtOH treatment down-regulated genes involved with the inflammatory response, defense response, response to wounding , chemotaxis, and IL-8-like chemokines.

The results also suggested that administering an ethanol extract from a different species (*E. paradoxa* rather than *E. angustifolia*) during day 6-8 post-infection again altered the pattern of gene expression. *Eang* H2O/*Epara* EtOH treatment up regulated 10 genes (Figure 10b and Table 3) and down regulated no genes compared to H2O/EtOH controls (Figure 11b and Table 5): PRLR (Prolactin receptor), Clcf1 (cardiotropin-like cytokine factor 1), Dock2 (Dedicator of cyto-kinesis 2), Ptprc (B220, Protein tyrosine phosphatase, receptor type, C), Vav1 (Vav1 Oncogene), IL5ra (IL-5 receptor alpha), Pik3cd (Phosphatidylinositol 3-kinase catalytic delta polypeptide), IL-27, Hdac5 (Histone deacetylase 5), and IL-11. DAVID analysis returned 2 annotation clusters >1.3 (Appendix). The evaluation of the GO terms data suggest that treatment with *Eang* H2O/*Epara* EtOH up regulated genes involved in the activation, differentiation of lymphocytes, leukocytes, and B-cells and up regulated genes involved Fcγ receptor-mediated phagocytosis.

The effect of day of post-infection on the gene expression is shown in figure 12. Genes up-regulated on day 8 were not identical to those up regulated on day 9 as expected with 63 genes up-regulated (Table 2) in both day 8 and day 9 groups treated with *Eang* H2O*/Eang* EtOH (Figure 12a). Similarly, on both day 8 and day9, both groups had 7 genes down regulated compared to uninfected mice (Figure 12b and Table 4); however, only 4 of these genes were shared between the days: Cr2, CXCR5, Ms4a1, and Wwp1. On day 8, CCL17, CD81, and Hdac5 were down regulated, and on day 9, the genes Jag2, IL4, and Hdac7 were down-regulated with *Eang* H2O*/Eang* EtOH treatment compared to the uninfected controls. Mice treated with *Eang* H2O*/Eang* EtOH to day 8 and day 9 compared to H2O/EtOH vehicle control demonstrated different genes up

regulated and down regulated with no gene regulation shared between the days when compared to the vehicle control (Table 3 and Table 5).

Discussion:

We have shown that although *Echinacea* combination treatment does not decrease the illness severity measures analyzed (weight loss and lesion scores), *Eang* H2O extract administered day 1-5 of influenza infection followed by *Eang* EtOH extract on days 6-9 of infection resulted in decreased BAL concentrations of cytokines that may have inflammatory effects (IL-6, IFN-γ and TNF-α). It was interesting to observe that anti-influenza IgG tended to be greater in the *Echinacea* treated mice, and this may reflect a shift away from a Th1 driven cell mediated immunity response (reduced IFN-γ and TNFα). The BAL concentration of several chemokines also tended to be decreased with *Echinacea* treatment compared to vehicle treatment, although this did not translate to altered numbers of specific cell subpopulations in the BAL. However, an analysis of cell populations at multiple time points would better detect changes in time that may have occurred with *Echinacea* extracts. In addition to these immunomodulatory changes, *Eang* H2O/*Eang* EtOH treatment resulted in up regulation of genes associated with cellular differentiation, activation, and proliferation of lymphocytes, monocytes, T cells and B cells, and down regulation of inflammatory cytokine/chemokine genes, and enhanced anti-influenza specific IgG production.

While it was hypothesized that treatment with a combination of *Echinacea* water and *Echinacea* ethanol extracts would decrease lung lesions seen in influenza mice at day 8 p.i., no significant difference was observed. In the *Eang* H2O/EtOH and *Eang*

H2O/*Eang* EtOH treatment, down regulation of genes associated with response to wound healing could explain the lack of change in lesion score and lung index; however, no change was also seen in the *Eang* H2O*/Epara* EtOH lesion score which did not have down-regulation in the wound healing associated genes. A possible explanation for this is the time point of this experiment being too early to detect visual changes between *Eang* H2O/*Epara* EtOH. With the down regulated wound healing response genes in *Eang* H2O/EtOH and *Eang* H2O/*Eang* EtOH, it is possible that no changes in lesion scores would be evident if down regulation of wound healing genes translates into a downstream effect of delayed wound healing. Other studies have shown that *Echinacea pallida* ethanol extract enhanced stress related wound healing in mice [16]. This punctuates the interspecies functional variability of *Echinacea* supplements.

Increased neutrophils present in the lung after injury or infection has been shown to promote acute lung injury and acute respiratory distress syndrome [20]. In severe cases of influenza infection, acute lung injury and secondary bacterial infections increase mortality. The down regulation of the IL-8-like chemokines genes (CCL3, CCL4, CCL5, CCL8) that are encoded for the chemokines involved in neutrophil recruitment to the lung at day 8, and the decrease in KC, LIX, RANTES, and MCP-1 in the BAL at day 9 could promote the decrease in acute lung injury in a severe influenza infection. The chemokines could be decreased in the lung at day 9 p.i. due to decreased gene expression of IL-8-like chemokine genes and down regulated wound healing response seen in the PCR data at day 8 during infection. A decrease in MCP-1 has been associated with delayed wound healing with decreased re-epithelialization [23]. However, increased MCP-1 has been shown to increase pulmonary fibrosis [24]. This suggests a role for

Echinacea in balancing the protective wound healing mechanisms in the lung. Lung injury resolution requires a balance of inflammation and fibroproliferation [25]. In the first experiment, LIX, MIP-2 and VEGF were decreased in the lung at day 9 in the mice treated with the *Echinacea* combination. LIX (CXCL5) and MIP-2 are neutrophil chemoattractants and VEGF is involved in wound healing. CXCL5 has been shown to be involved in lung inflammation in mice [26]. While it was hypothesized that the *Echinacea* extract combination would decrease the severity in lung lesions, the data presented in this study suggest the decrease in wound healing (based on gene expression, not actual histopathological differences). However, the down regulation in IL-8-like chemokine genes at day 8 could decrease the KC, RANTES, LIX, and MIP-2 thereby decreasing neutrophil recruitment, and prevent acute lung injury in a severe infection ultimately leading to decreased lesion scores and decreased neutrophil recruitment at a later time point.

Decreased production of IFN-γ, IL-6, TNFα, MCP-1 and RANTES in the lung detected in the BAL of *Echinacea* combination treated mice at day 9 could provide protection against lung inflammation. MCP-1 and RANTES have been shown to be involved in inflammation and the cellular infiltration of leukocytes into the lung in asthma [27]. The decrease in inflammation seen in both the BAL cytokine/chemokine data and lung gene regulation did not translate into decreased lung lesions at day 8 p.i. The time point selected in this study may have been too early to detect significant changes in the lung lesion scores.

The *Eang* H2O/*Epara* EtOH extract up regulated genes associated with lymphocyte/leukocyte activation and differentiation, B cell activation and FcγR-mediated

phagocytosis. Antigen-specific cytotoxic CD8 cells are important in influenza clearance, and gene expression of CD8 was observed. Increased T helper cell activation would provide enhancement to the humoral immunity and enhance influenza specific antibody production. The prolactin receptor (PRLR) signaling occurs through mechanisms similar to TCR signaling pathways by utilizing Fyn and Vav1 [28]. PRLR has been shown to be constitutively expressed on Treg populations [29] and prolactin signaling increases CD4 T-bet and Th1 response [30]. *Echinacea* has been shown to inhibit prolactin levels in serum [31]. However, it is possible that a compound in the extract modulates immune function through the PRLR. Future studies could investigate the role of PRLR immune signaling with *Echinacea*.

Antibodies in influenza both aid in the antibody-mediated neutralization and increased macrophage phagocytosis. In the *Eang* H2O/*Epara* EtOH treatment group, DAVID analysis suggested an up-regulation in the genes associated with FcγR-mediated phagocytosis. Although FcγR-mediated phagocytosis is associated with bacterial and fungal infections, a study utilizing FcγR knockout mice demonstrated that FcγR mediated phagocytosis was important for influenza viral clearance in vaccinated mice with increased mortality in the FcγR-/-mice upon secondary challenge with influenza [32]. Increased antibody mediated alveolar macrophage phagocytosis of influenza virus was also observed in a phagocytic assay [32]. Another study demonstrated that the impairment of macrophage at late phase of infection resulted in greater susceptibility to secondary bacterial infections [33]. Enhanced macrophage phagocytosis has been shown with *Echinacea*. Polysaccharides isolated from botanical extracts have demonstrated a variety of effects on macrophage activation with enhanced phagocytosis and NO

production and decreased inflammatory cytokine production [34,35]. Alveolar macrophage enhanced phagocytosis was also shown in chicoric acid, polysaccharides, and alkamide fractions of *Echinacea purpurea* [36]. Although in our study macrophage cellular counts were not changed between the *Eang* H2O/*Epara* EtOH compared to and H2O/EtOH treatment, it is still feasible that the overall activation of the macrophages could potentially be enhanced. The *Eang* H2O/*Epara* EtOH combination treatment could potentially promote clearance of virus and cellular debris through enhanced FcγR mediated phagocytosis in macrophages.

Echinacea water extracts have an immunostimulatory property that is conferred largely through hydrophilic polysaccharides that are present in the water based preparations. These polysaccharides have been shown to stimulate the innate immune system. In one study human white blood cells were harvested and stimulated using several water *Echinacea* polysaccharide preparations and were found to be stimulated in the presence of the extract compared to media treatment [15]. *Echinacea* water-based extracts have been shown to increase number and functions of NK cells and activation and maturation of DCs [13,14]. Activation of NK cells and maturation of DCs are necessary for viral clearance and the recovery from influenza infection. *Echinacea* extracts have been shown to have anti-viral properties *in vitro,* but have not been demonstrated to have clearly anti-viral effect in an *in vivo* model. Polysaccharides and chicoric acid are potential compounds present in *Echinacea* extracts that have been identified as demonstrating an anti-viral effect. It is possible that the early treatment with *Eang* H2O based extract polysaccharides or chicoric acid could be responsible for the trend in viral titer reduction that was observed in the *Eang* H2O*/Eang EtOH* treated mice

compared to the H2O/EtOH treated mice. Arabinogalactan is one of the polysaccharides that have been isolated from *Echinacea* water preparations. In a study with arabinogalactan isolated from Larch tree and not *Echinacea*, increased levels of IgG antibodies to *Streptococcus pneumonia* were demonstrated in the serum of humans [37]. Arabinogalactan in the *Eang* H2O preparation used in this study could be responsible for the trend towards an increase in anti-influenza specific antibody seen in the *Eang* H2O/*Eang* EtOH treated mice compared to the H2O/EtOH control mice. Further study with arabinogalactan isolated from *Eang* would be required to confirm this hypothesis.

Another possible explanation for the decrease in viral titer is the modulation of the cellular adaptive immune pathways. An increase in the plasmacytoid dendritic cells (pDC) population and enhancement through cellular adaptive immune pathways would be another possible explanation for the trend towards an increase in anti-influenza antibody production by the *Eang* H2O/*Eang* EtOH treated mice through pDC dependent B cell activation. Mucosal pDCs produce type I IFN and have been shown to aid in viral clearance through pDC-dependent antibody production [38]. Further study of the pDC production of type I IFN from mice treated with *Echinacea* would be needed to determine if this mechanism would be responsible for the reduction in viral titer.

Alkamides from *Echinacea* plants have been shown to be responsible for the anti-inflammatory properties associated with *Echinacea* extracts. Alkamides are the primary active constituent in ethanol-based extracts of *Echinacea*. A decrease in inflammatory cytokines in this study was shown in both the BAL of mice treated with *Eang* H2O/*Eang* EtOH and in the gene expression profiles reflecting down-regulation in several inflammatory cytokine/chemokines. This gene down regulation and decrease in

inflammatory cytokines did not translate into decreased lesion scores or in decreases in BAL cell populations. One possible explanation for this observation is that the cell populations that are decreasing in the lung were not specifically analyzed, such as NK cells or T cell subtypes. NK cells and cytotoxic T-cells are essential for viral clearance but can also add to the immunopathology of influenza infection, particularly NK cells that have been shown to enhance lung inflammation [39]. *Echinacea* alkamides have been shown to inhibit inflammatory cytokine production in RAW264.7 murine macrophages in response to LPS and influenza A/PR/8/34 [40,41]. TNF-α was shown to be decreased in a dose dependent manner [41]. IL-10 and KC were decreased in the lungs and IFN-γ was decreased in the serum of C57BL/6 mice infected with influenza A/WSN/33 when treated with polysaccharide extracts from *E. purpurea* [42]. Similar decreases in cytokines and chemokines were observed in *E. angustifolia* and *E. pallida* ethanolic extracts administered *in vivo* to BALB/c mice which demonstrated decreased TNF-α and IL-1β with increased IL-10 in splenocytes stimulated *in vitro* after 7 days of treatment [12]. Caffeic acid derivatives have also been shown to have anti-inflammatory effects, but the effects are dampened compared to the anti-inflammatory effects seen with the alkamide fractions [40]. Caffeic acid derivatives were shown to increase activation of iDCs through increased CD83 expression in an *E. purpurea* butanol extract applied to DCs cultured *in vitro* [43]. The decrease in anti-inflammatory cytokines is not surprising in this study; however, the lack of change in the cellular populations was unexpected. Based on the cellular populations tested, we expected a decrease in neutrophils and macrophages. However, with decreased RANTES and eotaxin the cell populations

decreased in response to the cytokine profile may be NKs, DCs, eosinophils, and subtypes of T-cells.

There have been some questions surrounding the bioavailability of the active constituents in *Echinacea* extract preparations. Alkamides and caffeic acid have been demonstrated to be readily absorbed and bioavailable through CACO-2 monolayers and human studies evaluating the absorption in serum samples and through excretion [44,45]. Other questions surrounding *Echinacea* preparations include the anti-inflammatory effect due to the ethanol and immune-stimulatory effect due to endotoxins [46]. In this study, we used extracts prepared in water free of endotoxins and controlled for ethanol effect by utilizing the H2O/EtOH control treatment group which included the same concentration of ethanol that was present in the *Echinacea* extracts. Invariably, endotoxin is present in *Echinacea* root preparations, particularly those prepared in water, such as a tea preparation [46,47]. Typical endotoxin mediated immune responses include, increases in toll-like receptors, production of inflammatory factors, and macrophage activation. These hallmarks were not observed at the time point measured in this study. However, it will be essential determine whether endotoxin was present in the extracts, and if so, at what concentration. While this study did not evaluate the effect of the combination of extracts on the immune response uninfected mice, other studies have reported a general immunostimulatory response. Several studies have analyzed the components found in *Echinacea* extracts; however, the mechanisms behind the complex complete extract and interaction of constituents remains elusive. Studies utilizing fractions of extracts could be beneficial in extrapolating these mechanisms.

This study is the first to explore the utilization of combinations of *Echinacea* treatments to enhance the immune response and resolve influenza infections. To our knowledge, it is also the first *Echinacea* study to utilize DAVID analysis of immune gene regulation in influenza infection. The different expression profiles from the *Echinacea* treatment groups revealed suggest that the combination selected could greatly impact the anti-inflammatory effects in the lung. No significant treatment effects were observed with respect to the disease severity measures, body weight, or lesion scores. However, there was a trend towards reduced viral load in the mice treated with the *Echinacea* combination at day 9. Although, the immunomodulatory effect is determined by *Echinacea* species utilized, the data in this study suggest the possibility of enhancing the innate and cellular immune response particularly macrophage phagocytosis, lymphocyte activation and differentiation, along with increased antibody production by utilizing an *Echinacea* water extract at the early phase of infection. Also, the data in this study support a decrease in pro-inflammatory cytokines, chemokines responsible for neutrophil recruitment, and down-regulation of genes associated with the wound response and neutrophil recruitment during later phases of infection using an anti-inflammatory *Echinacea* ethanol extract during the late phase of influenza infection.

Figures for Chapter III.

Figure 1.

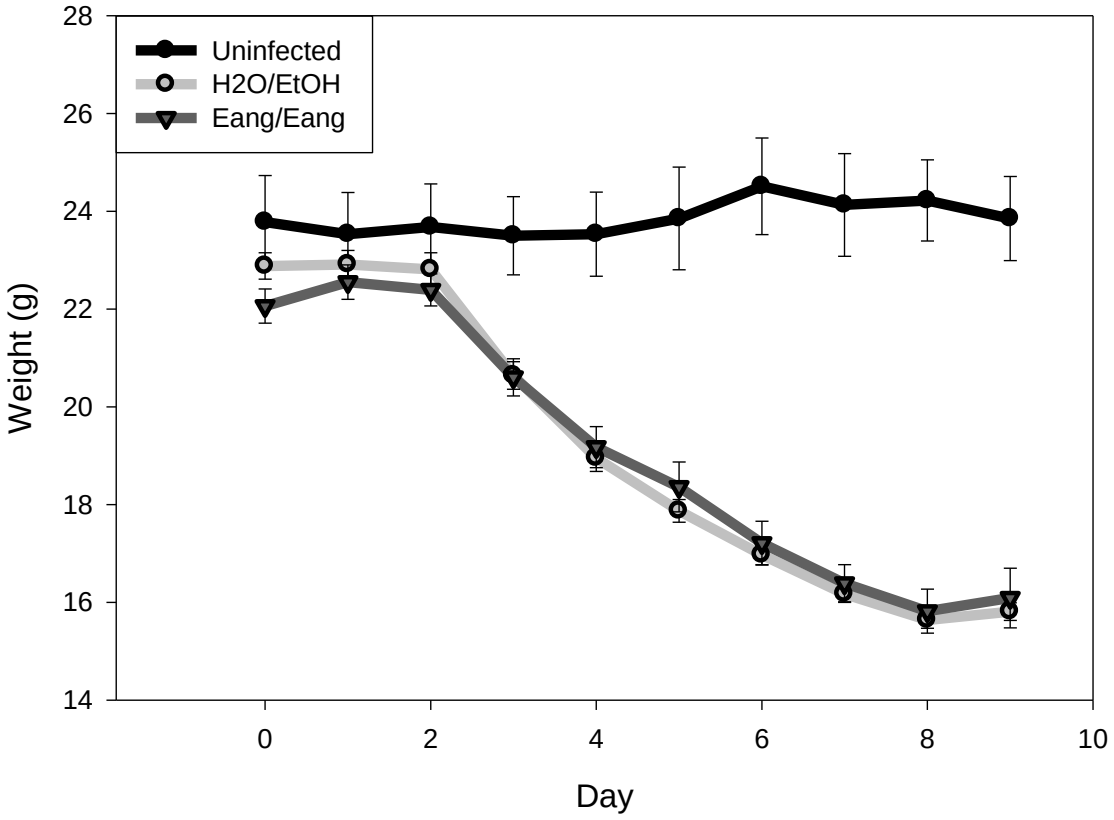

Figure 1. Experiment 1 Illness Severity Measure: Daily weights post infection with A/PR/8/34. Mice were gavaged with either *Echinacea angustifolia* (*Eang*) water extract or H2O control for one day prior to intranasal infection with 0.001 HAU A/PR/8/34 through day 5p.i. Mice were then gavaged with either *Eang* ethanol extract (instead of *Eang* water) or EtOH control (instead of H2O) until day 9p.i. There was no significant difference between the *Eang* H2O/*Eang* EtOH treatment mice compared to the H2O/EtOH control mice. Measures are given as means +/- standard error of the mean (SEM). n=3 uninfected; n=6 H2O/EtOH; n=9 *Eang* H2O/*Eang* EtOH.

Figure 2.

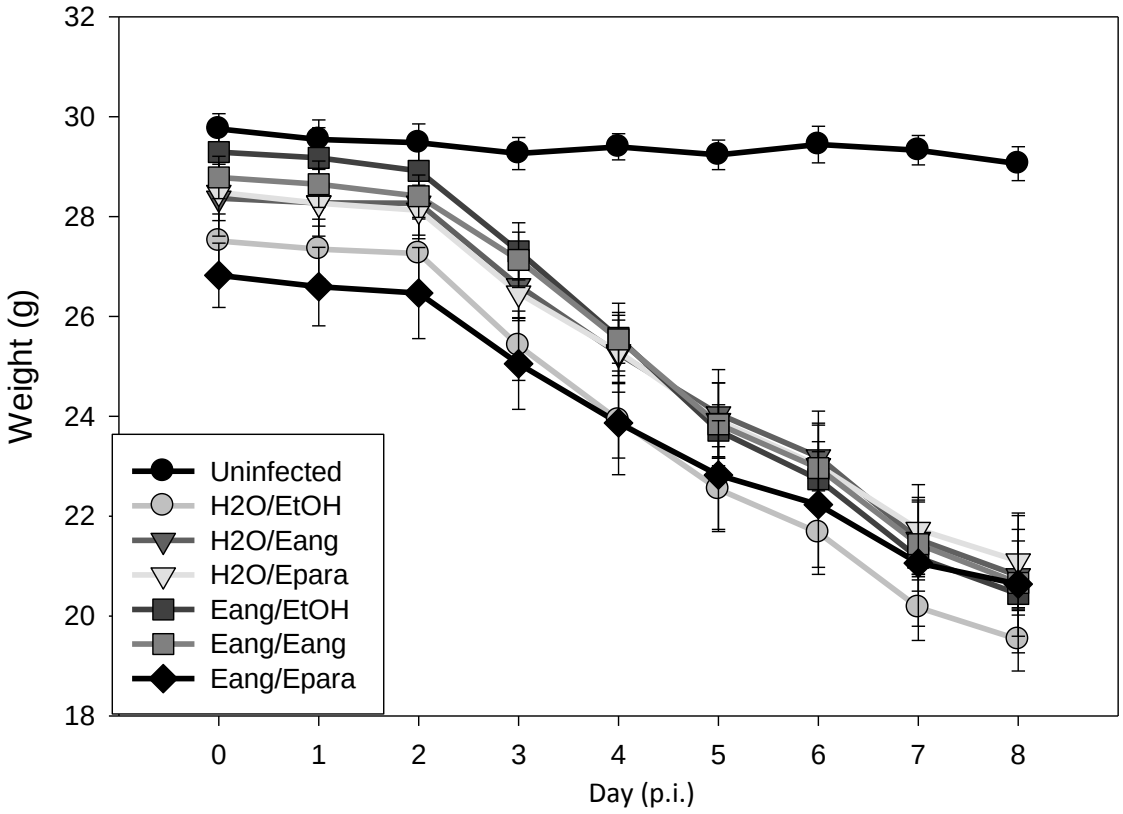

Figure 2. Experiment 2 Illness Severity Measure: Daily weights post infection with A/PR/8/34. Mice were gavaged with either *Echinacea angustifolia* (*Eang*) water extract or H2O control for one day prior to intranasal infection with A/PR/8/34 through day 5 p.i. Mice were then gavaged with one of the *Echinacea* ethanol extracts (*Eang-E. angustifolia* or *Epara-E. paradoxa var. paradoxa*) or EtOH control until day 8 p.i. Measures are given as means +/-SEM. n=4 uninfected; n=10 H2O/EtOH; n=10 H2O/*Eang* EtOH; n=12 H2O/*Epara* EtOH; n=8 *Eang* H2O/EtOH; n=9 *Eang* H2O/*Eang* EtOH; n=8 *Eang* H2O/*Epara* EtOH.

Figure 3.

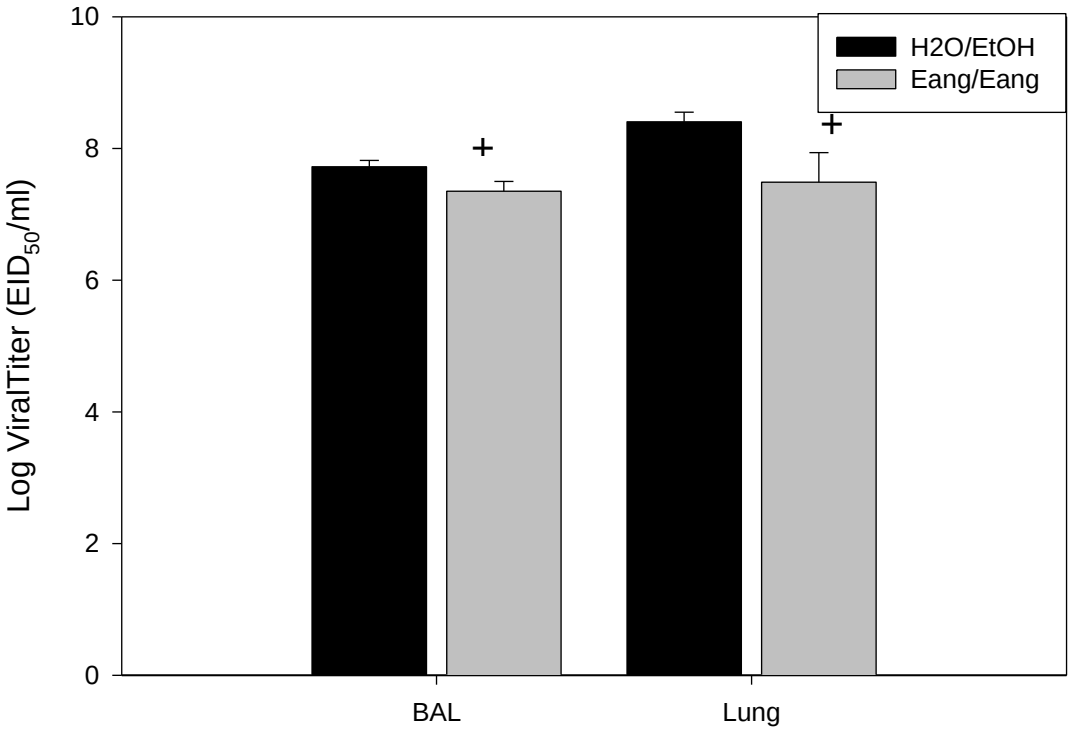

Figure 3. Lung Viral titers of influenza assessed by polymerase chain reaction in mice infected with A/PR/8/34 at 9 days p.i. and treated with a combination of *Eang* extracts or control. BALB/c mice were infected with A/PR/8/34 intranasal and treated with either H2O/EtOH or *Eang* H2O/*Eang* EtOH. Mice treated with *Eang* water for 5 days p.i. followed by *Eang* EtOH from day 6-9p.i. had a trend towards decreased virus in the BAL and in the lung (p=0.08) as indicated by +. Measures are given in Log 10 of the mean viral titer as determined by PCR +/- SEM. . n=6 H2O/EtOH; n=9 *Eang* H2O/*Eang* EtOH)

Figure 4a.

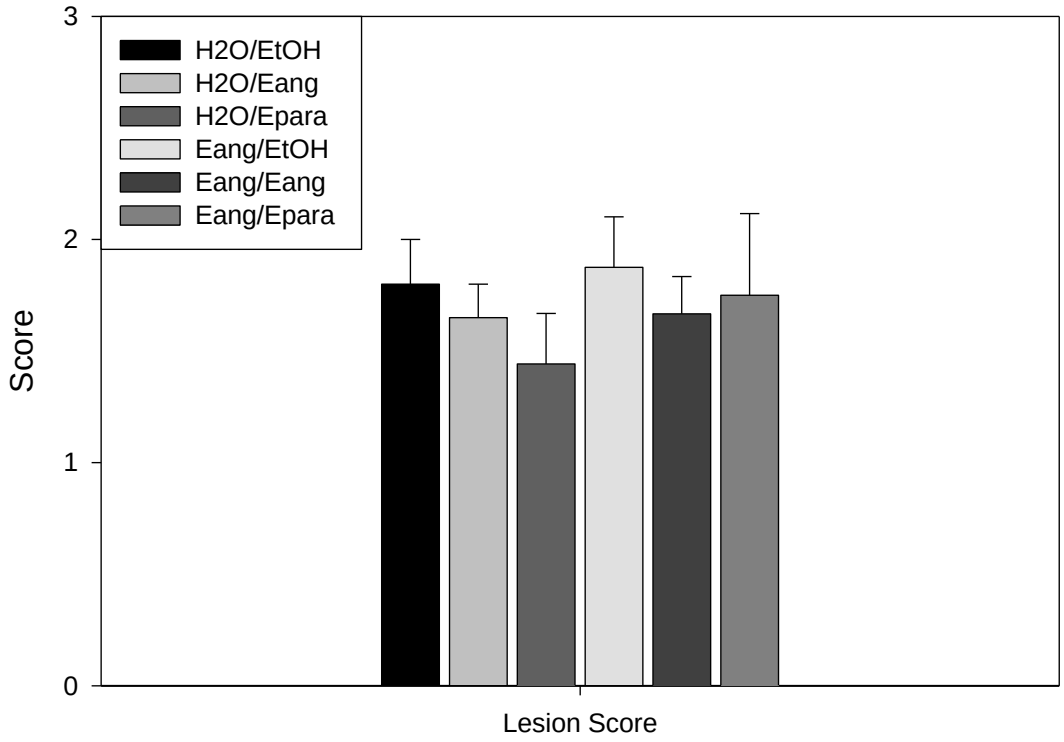
3
2
1
0
Score
H2O/EtOH
H2O/Eang
H2O/Epara
Eang/EtOH
Eang/Eang
Eang/Epara
Lesion Score

Figure 4b.

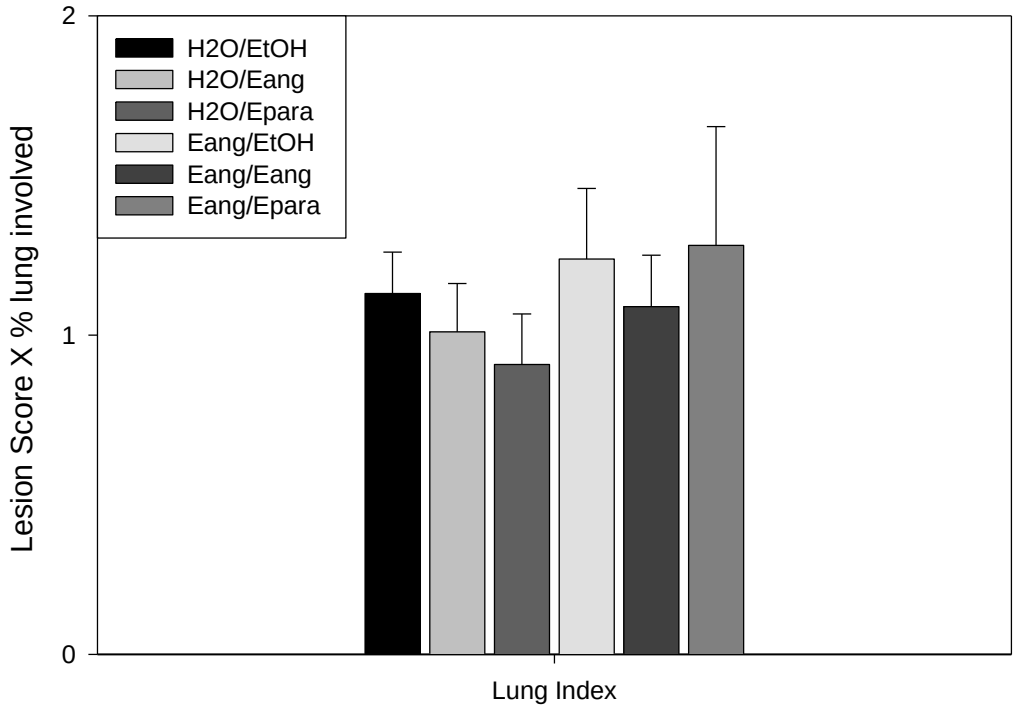

Figure 4a, b. Lung lesion evaluation 8d p.i. with *Echinacea* combination treatments or control. Mice were treated with a combination of water (day 1-5 p.i.) and ethanol extracts (day 6-8 p.i.). Lungs were collected on day 8 p.i. Tissue sections were analyzed by a pathologist, and influenza induced pathology was assessed. Lesion scores were determined by the size and extent of the pathology. A.) Lesion scores. Results are given as mean lesion score for each treatment group +/- SEM. There was no statistical difference observed in the lesion scores in the lungs of mice treated with the *Echinacea* combination compared to H2O/EtOH control. B.) Lung index was determined by multiplying the lesion score by the percent of the lung involved. There was no statistical difference between the lung index scores of combination treated mice compared to H2O/EtOH. n=4 uninfected; n=10 H2O/EtOH; n=10 H2O/*Eang* EtOH; n=12

H2O/*Epara* EtOH; n=8 *Eang* H2O/EtOH; n=9 *Eang* H2O/*Eang* EtOH; n=8 *Eang* H2O/*Epara* EtOH.

Figure 5a.

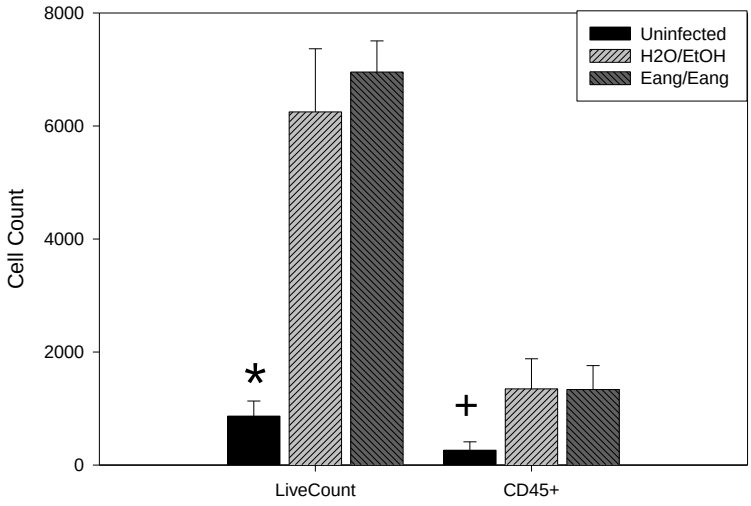

Figure 5b.

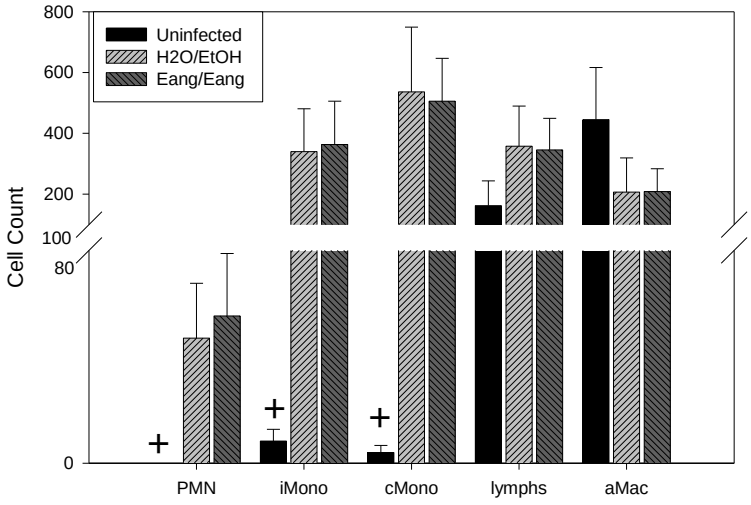

Figure 5c.

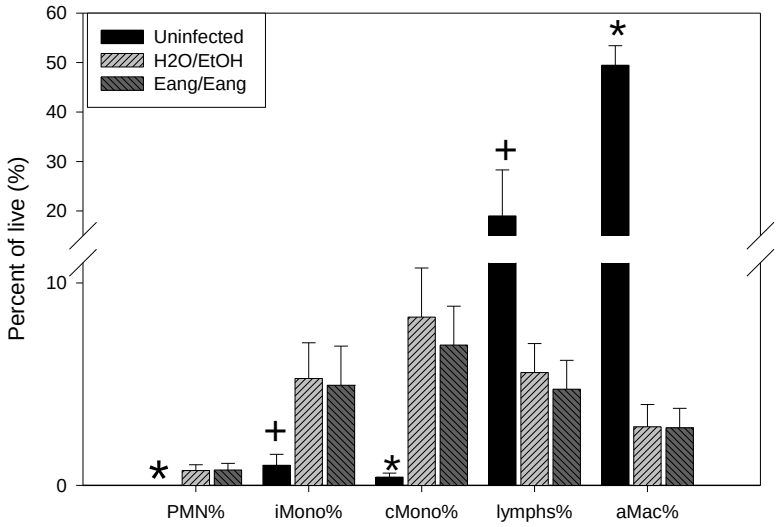

Figure 5a, b, c. Effect of *Eang* H2O/*Eang* EtOH treatment of mice on cellular infiltration of the lung compared to vehicle control treatment. Mice were treated with *Eang* H2O for 5 days p.i. then with *Eang* EtOH for day 6-9 p.i. Cells were isolated from BAL at 9 days p.i. from *Eang* H2O/*Eang* EtOH or H2O/EtOH treated mice infected with influenza and analyzed by flow cytometry for cell populations. A.) Cell counts are given for total live cells and CD45+ cells. Measures given as mean +/-SEM. B.) Cell counts for populations analyzed, measured as mean +/-SEM. C.) Populations in B given as % total live cells in BAL +/- SEM. No statistical difference was observed in the BAL cell populations comparing *Eang* H2O/*Eang* EtOH to the vehicle control. Cell populations were significantly different between the mice treated with H2O/EtOH compared to the uninfected control as indicated by *, $p<0.05$, or +, $p=0.1$ by student t-test. n=3 uninfected; n=6 H2O/EtOH; n=9 *Eang* H2O/*Eang* EtOH.

Figure 6a.

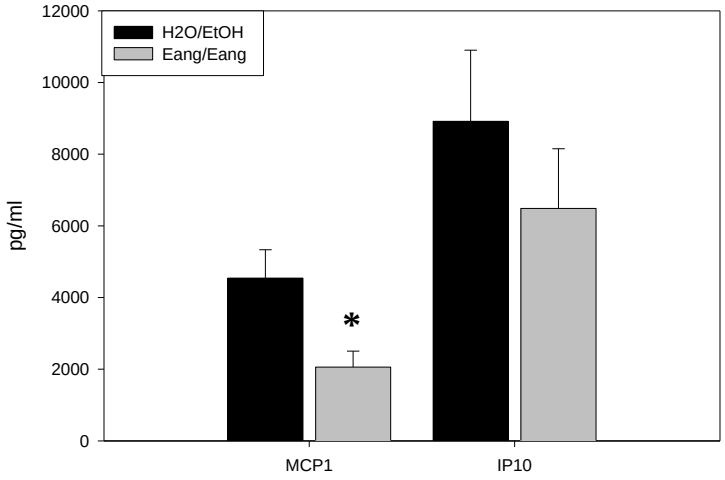

Figure 6b.

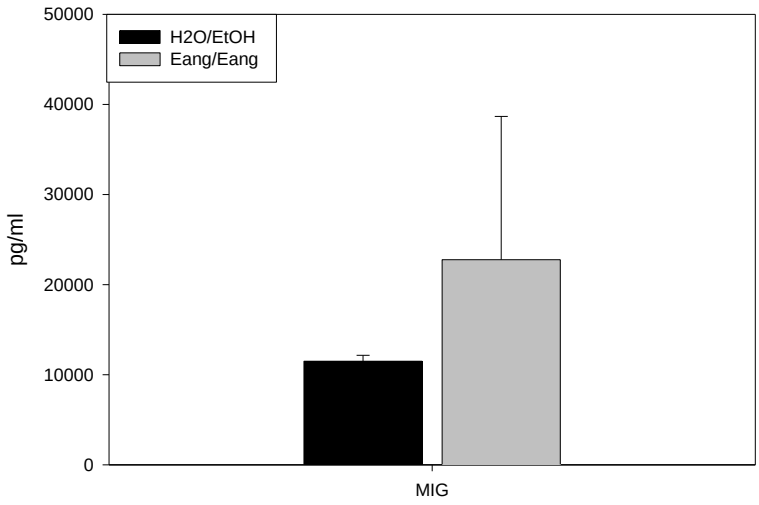

Figure 6c.

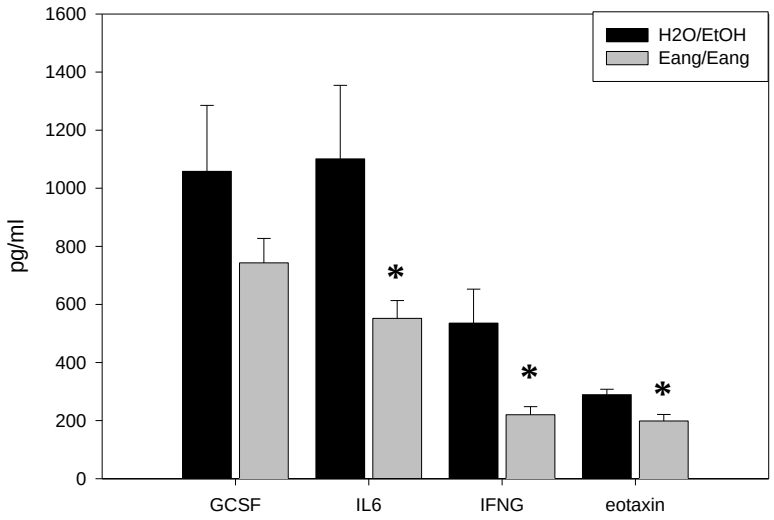

Figure 6d.

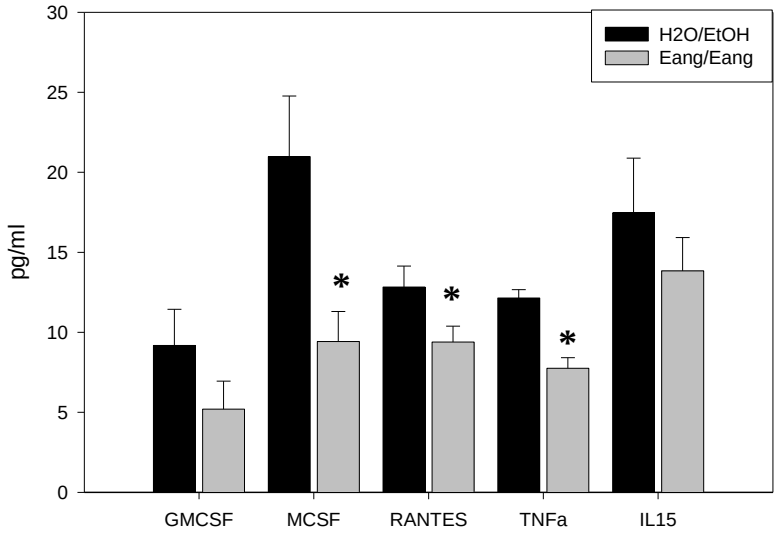

Figure 6e.

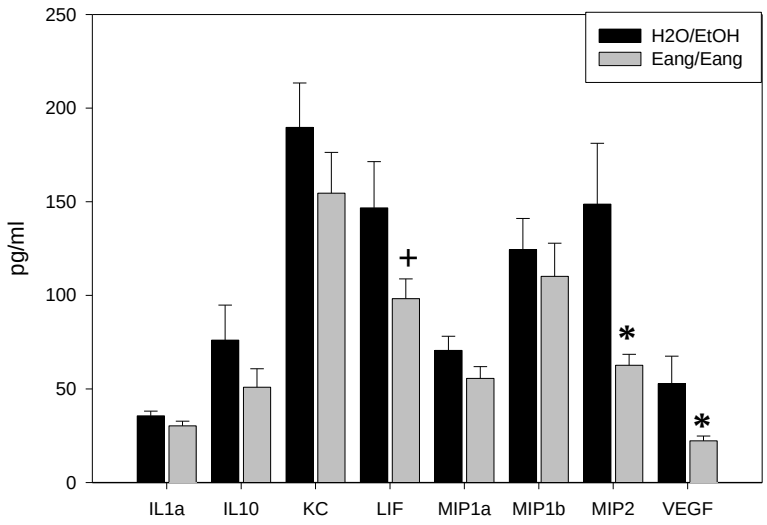

Figure 6f.

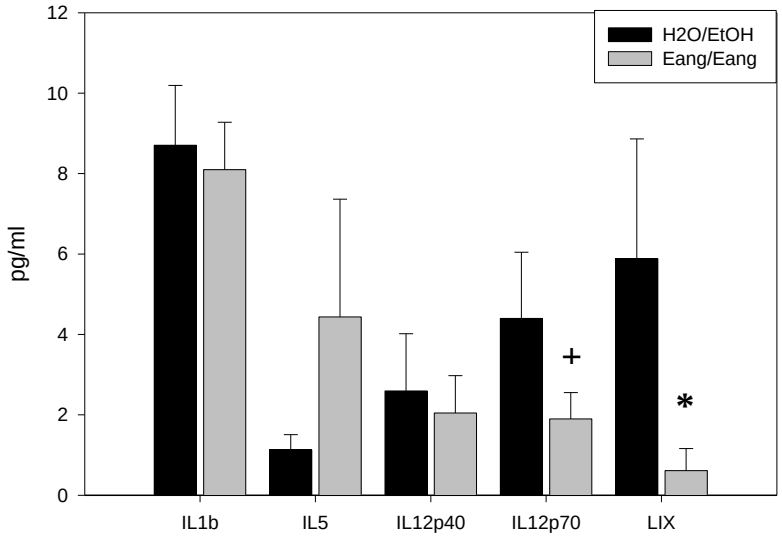

Figure 6a-f. Cytokines/Chemokines in BAL day 9 p.i. from mice infected with influenza. BAL fluid was collected and analyzed using Bio-plex® multiplex testing for cytokines and chemokines at day 9 p.i. Cytokines/Chemokines figures are shown in order based on concentration with highest concentration shown first. Decreases in Eotaxin ($p<0.05$), IFN-γ ($p<0.05$), IL-6 ($p<0.05$), IL-12p70 ($p=0.1$), LIF ($p=0.06$), LIX ($p<0.05$), MCP-1 ($p<0.05$), M-CSF ($p<0.05$), MIP-2 ($p<0.05$), RANTES ($p<0.05$), TNF-α ($p<0.05$), and VEGF ($p<0.05$) were observed in the *Eang* H2O/*Eang* EtOH treatment group compared to the H2O/EtOH vehicle control. Measures are mean +/-SEM. Student t-test was used to evaluate the *Eang* H2O/*Eang* EtOH treatment against the vehicle control. *, $p<0.05$; +, $p<0.1$ n=6 H2O/EtOH; n=9 *Eang* H2O/*Eang* EtOH.

Figure 7.

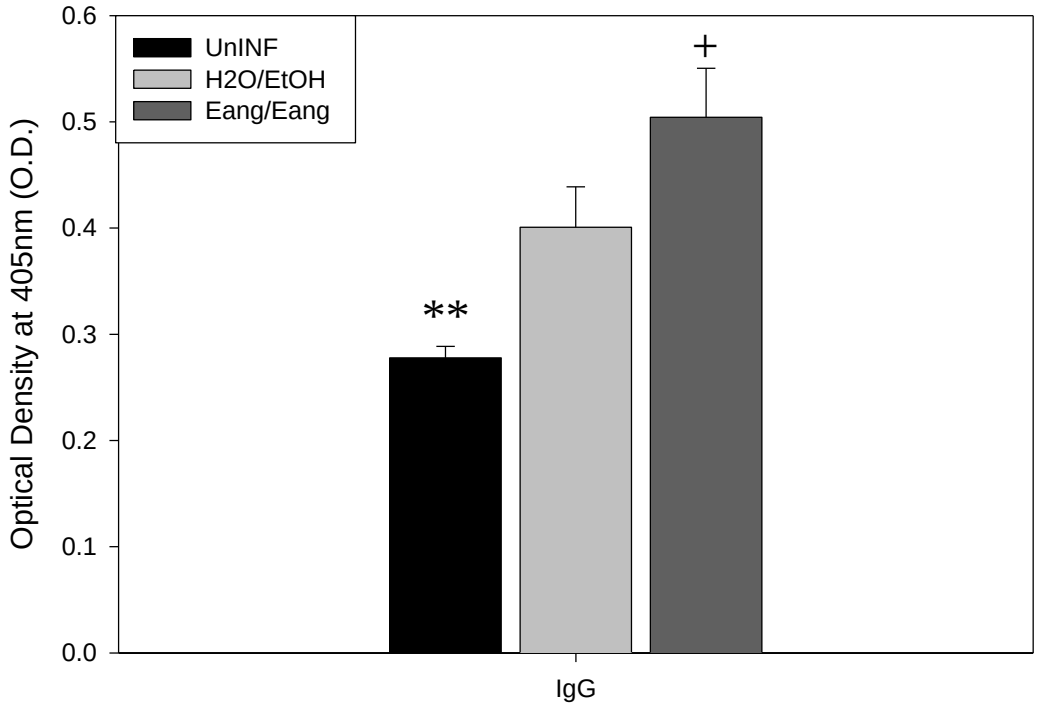

Figure 7. Effect of *Echinacea* combination treatment on Anti-influenza IgG production at day 9 p.i. Mice were gavaged for 1 day prior to infection with influenza A/PR/8/34 and 5 days post-infection (p.i.) with *Eang* water extract or H2O control followed by *Eang* EtOH or EtOH control until day 9 p.i. Serum was collected on day 9 p.i. and tested for Anti-influenza IgG by enzyme linked immunoassay (ELISA). Optical density (O.D.) demonstrated by the uninfected mice represents the background in the assay. Both H2O/EtOH and *Eang* H2O/*Eang* EtOH had O.D. values greater than the background ($p<0.05$). H2O/EtOH treated mice demonstrated a lower O.D. reading than the *Eang H2O/Eang EtOH* ($p=0.06$) indicating that H2O/EtOH had lower anti-influenza IgG present in their serum compared to the *Eang* H2O/*Eang* EtOH treated mice. Results

are given as average O.D. +/-SEM. +, p=0.06 (n=6 for H2O/EtOH; n=9 for *Eang* H2O/*Eang* EtOH).

Figure 8.

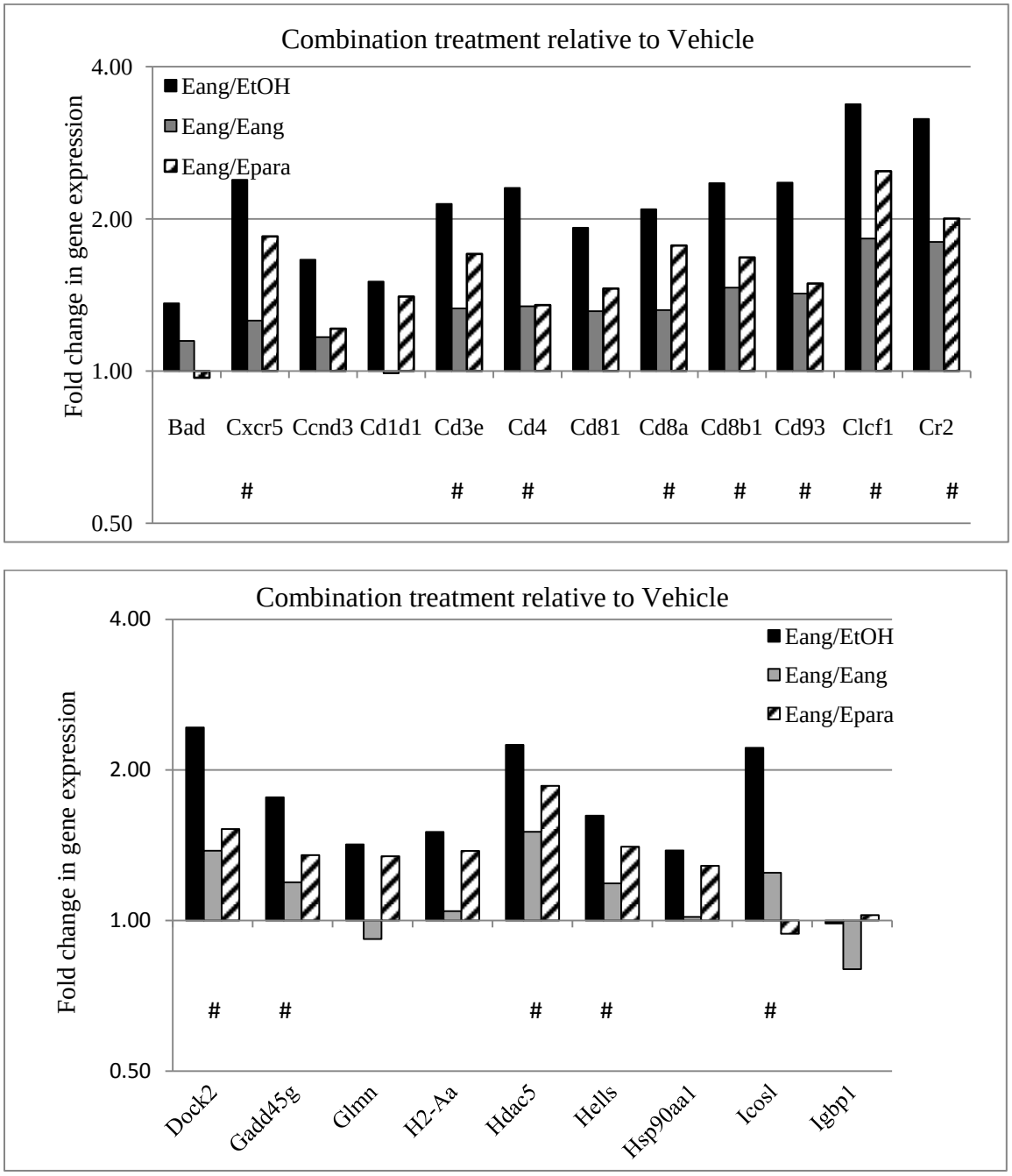

Figure 8. continued.

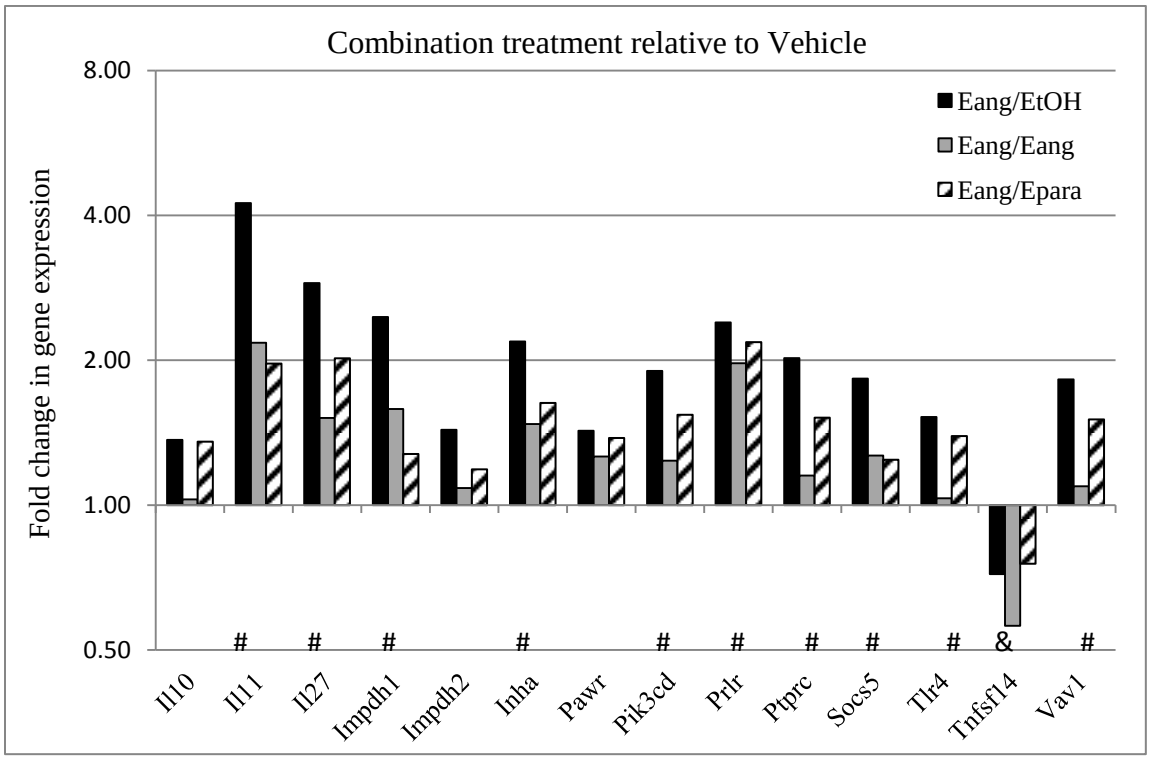

Figure 8. Effect of *Echinacea* combination treatments on gene expression in lungs at day 8 p.i. T and B cell array gene fold regulation combination treatment relative to vehicle treatment measured by q-rtPCR microarray from cDNA isolated from lungs of mice treated with a combination of extracts and infected with influenza A/PR/8/34. Genes shown were significantly changed with extract combination treatment $p<0.1$. Day 8 only. H2O/EtOH n= 6; *Eang H2O/*EtOH n=5; *Eang* H2O/*Eang* EtOH n= 7; *Eang H2O/Epara* EtOH n=6. Genes that were selected by DAVID analysis to be >1.5 fold change (#) or <0.667 (&) with fold enrichment.

Figure 9.

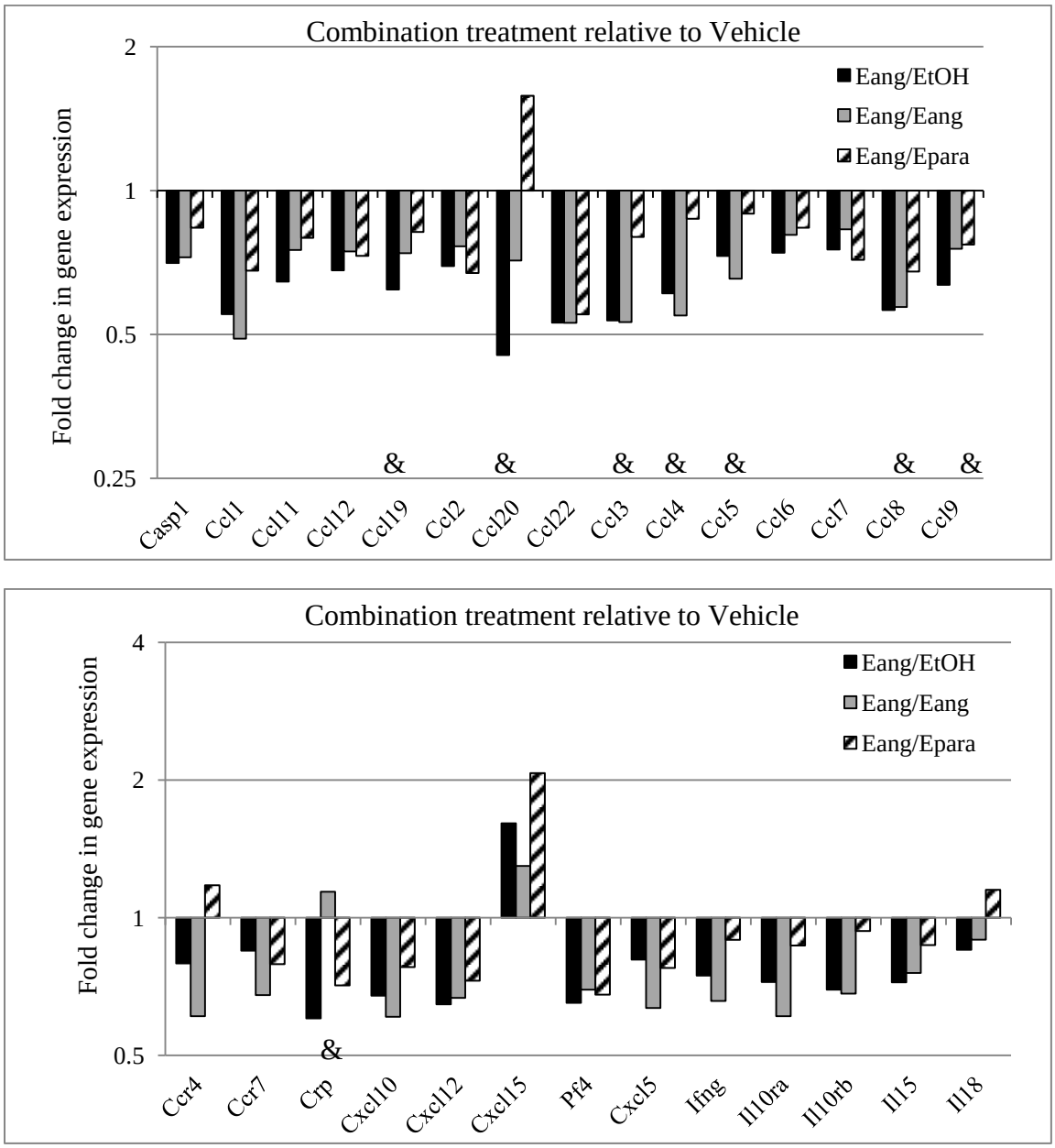
Combination treatment relative to Vehicle
Fold change in gene expression
2
1
0.5
0.25
Eang/EtOH
Eang/Eang
Eang/Epara
&
Casp1
Ccl1
Ccl11
Ccl12
Ccl19
Ccl2
Ccl20
Ccl22
Ccl3
Ccl4
Ccl5
Ccl6
Ccl7
Ccl8
Ccl9
Combination treatment relative to Vehicle
Fold change in gene expression
4
2
1
0.5
Eang/EtOH
Eang/Eang
Eang/Epara
&
Ccr4
Ccr7
Crp
Cxcl10
Cxcl12
Cxcl15
Pf4
Cxcl5
Ifng
Il10ra
Il10rb
Il15
Il18

Figure 9. continued.

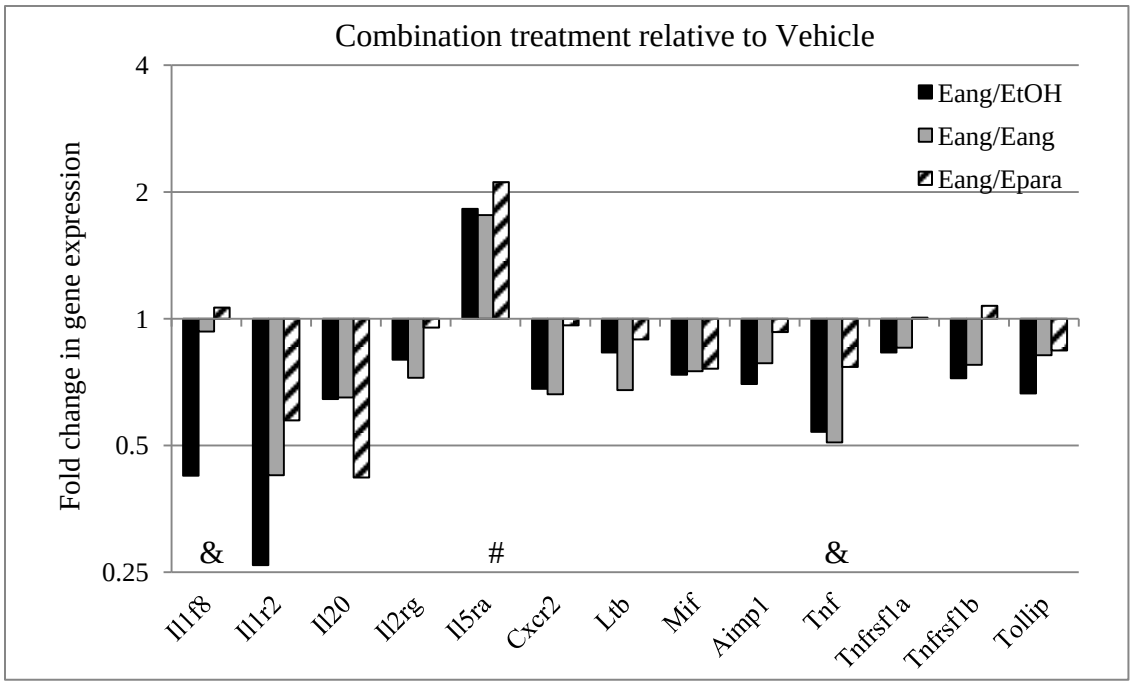

Figure 9. Effect of *Echinacea* combination treatments on gene expression in lungs at day 8 p.i. Cytokine and Chemokine array gene fold regulation combination treatment relative to vehicle treatment measured by PCR microarray from cDNA isolated from lungs of mice treated with a combination of extracts and infected with influenza A/PR/8/34. Day 8 only. H2O/EtOH n= 6; *Eang H2O/*EtOH n=5; *Eang* H2O*/Eang* EtOH n= 7; *Eang H2O/Epara* EtOH n=6. Genes that were selected by DAVID analysis to be >1.5 fold change (#) or <0.667 (&) with fold enrichment.

Figure 10a. – Day 8 p.i., number of genes up regulated relative to uninfected

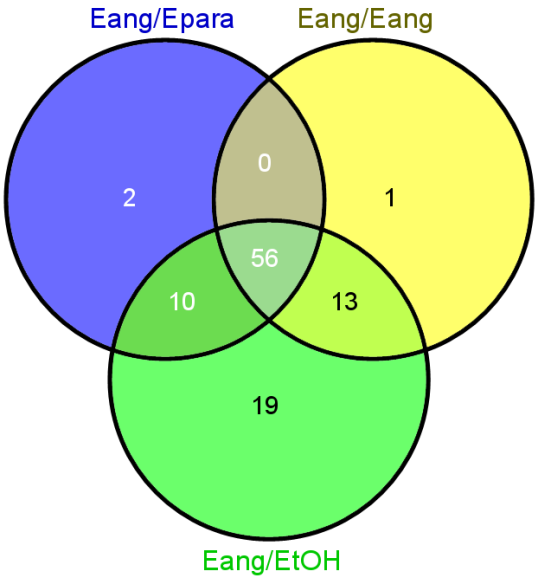

Figure 10b. –Day 8 p.i., number of genes up regulated relative to H2O/EtOH

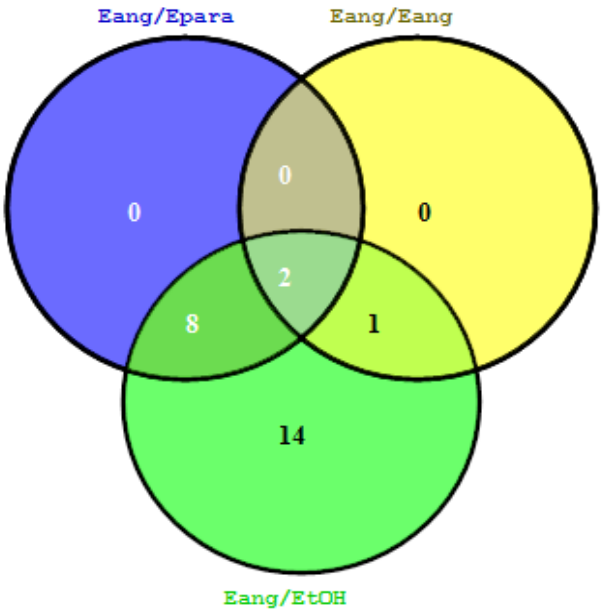

Figure 10a-b. Venn diagrams illustrating the *Echinacea* combination treatment gene up-regulation profiles for Day 8 treatment groups. Venn diagrams were created to illustrate the gene up regulation in the DAVID fold enrichment. These diagrams magnify the different gene regulation profiles demonstrated by each treatment group. A.) *Echinacea* combination groups compared to uninfected mice (effect of infection with the treatment). *Eang* H2O/EtOH up-regulated a total of 98 genes, *Eang* H2O/*Eang* EtOH up-regulated 70, *Eang* H2O/*Epara* EtOH up-regulated 68 genes. 56 genes were up regulated similarly in all three treatment groups. 19 genes were up-regulated only by *Eang* H2O/EtOH treatment; whereas, *Eang* H2O/*Eang* EtOH up regulated 1 gene differently than the other treatments and *Eang* H2O/*Epara* EtOH up regulated 2 genes differently than the other treatment groups. B.) *Echinacea* combination groups compared to H2O/EtOH controls (effect of combination treatment). *Eang* H2O/EtOH up-regulated a total of 25 genes, *Eang* H2O/*Eang* EtOH up-regulated 3, *Eang* H2O/*Epara* EtOH up-regulated 10 genes. 14 of these genes were not up regulated by the other two treatment groups. 2 genes were shared with both *Eang* H2O/*Eang* EtOH and *Eang* H2O/*Epara* EtOH. 8 genes were up regulated in both *Eang* H2O/EtOH and *Eang* H2O/*Eang* EtOH; whereas 1 gene was up regulated in both *Eang* H2O/EtOH and *Eang* H2O/*Epara* EtOH. Neither *Eang* H2O/*Eang* EtOH nor *Eang* H2O/*Epara* EtOH up-regulated genes that was not also up-regulated with one of the other treatment groups.

Figure 11a. – Day 8 p.i., number of genes down regulated relative to uninfected

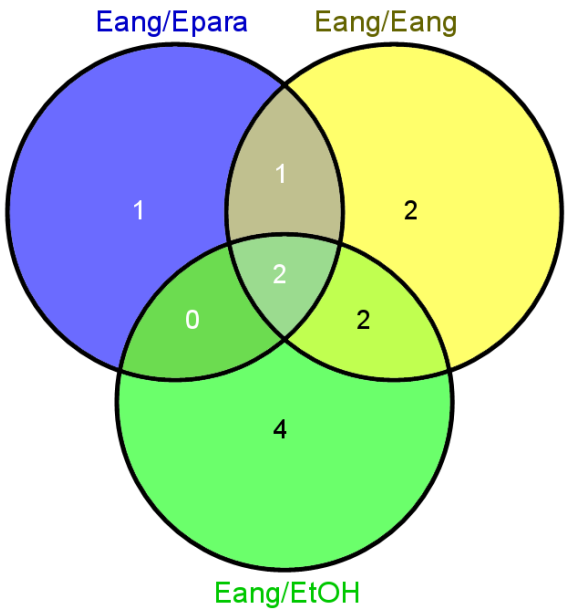

Figure 11b. – Day 8 p.i., number of genes down regulated relative to H2O/EtOH

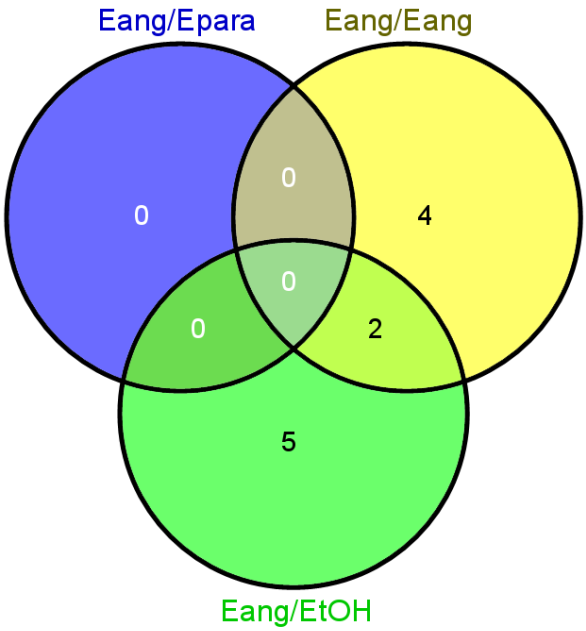

Figure 11a-b. Venn diagrams illustrating the *Echinacea* combination treatment gene down-regulation profiles for Day 8 treatment groups. Venn diagrams were created to illustrate the gene down regulation in the DAVID fold enrichment. A.) *Echinacea* combination groups compared to uninfected mice (effect of infection). *Eang* H2O/EtOH down regulated a total of 8, *Eang* H2O/*Eang* EtOH down regulated 7, and *Eang* H2O/*Epara* EtOH down regulated 4 in respect to infection. 2 of these genes were down regulated in all 3 treatment groups. 4 were exclusively down regulated in *Eang* H2O/EtOH, 2 exclusively down regulated in *Eang* H2O/*Eang* EtOH treatment and only 1 exclusively in *Eang* H2O/*Epara* EtOH. B.) *Echinacea* combination groups compared to vehicle control (H2O/EtOH) to illustrate effect of extract combination. *Eang* H2O/EtOH down regulated a total of 7, *Eang* H2O/*Eang* EtOH down regulated 4, and *Eang* H2O/*Epara* EtOH down regulated 0 genes in respect to H2O/EtOH controls. *Eang* H2O/EtOH *and Eang* H2O/*Eang* EtOH both down regulated 2 of the same genes compared to H2O/EtOH controls.

Figure 12a.- comparison of infection effect on day 8 and day 9 p.i. with the same extract treatment, up regulated relative to uninfected

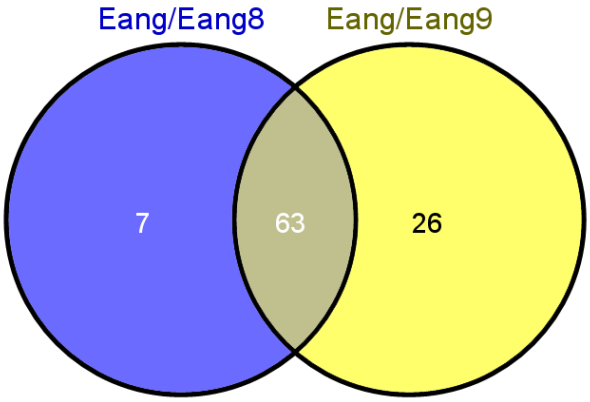

Figure 12b.- comparison of infection effect on day 8 and day 9 p.i. with the same extract treatment, down regulated relative to uninfected

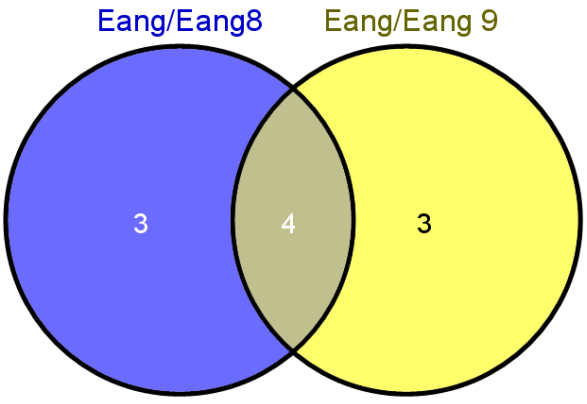

Figure 12a-b. Effect of day post infection on gene regulation. Venn diagrams comparing gene regulation between the *Eang* H2O/*Eang* EtOH day 8 and *Eang* H2O/*Eang* EtOH day 9. Venn diagrams were created to illustrate the gene up regulation in the DAVID fold enrichment between day 8 and day 9. A.) *Eang* H2O/ *Eang* EtOH day 8 and day 9 up-regulated compared to uninfected mice (effect of infection). On day 8, 70 genes were up regulated by treatment compared to uninfected mice. On day 9, 89 genes were up-regulated. 63 of these genes were up regulated on day 8 and day 9. B.) *Echinacea* day 8 and day 9 down-regulated genes compared to uninfected mice to demonstrate the *Echinacea* treatment specific changes between day 8 and day 9. Both day 8 and day 9 *Eang* H2O/ *Eang* EtOH treatment down regulated 7 genes; however the same 7 genes were not down regulated. 4 genes were down regulated similarly in both day 8 and day 9; whereas, 3 genes were exclusive to each day.

Table 1. Effect of infection and extract treatment on lung cytokine/chemokines

	Uninfected		**H2O/EtOH**			***Eang* H2O/*Eang* EtOH**		
N=	3		6		vs. Uninfected	9		vs. Vehicle
	Mean	SEM	Mean	SEM	P-value	Mean	SEM	P-value
Eotaxin	3.13	0.98	[a]289.30	18.55	0.00	*198.73	22.14	0.01
G-CSF	0.59	0.59	[a]1058.06	227.05	0.01	742.94	84.16	0.16
GM-CSF	0.00	0.00	[a]9.17	2.26	0.01	5.20	1.75	0.18
IFN-γ	7.08	0.89	[a]535.39	117.01	0.02	*220.16	27.73	0.04
IL-1α	34.86	2.01	35.63	2.47	0.85	30.22	2.55	0.17
IL-1β	12.30	2.59	8.71	1.49	0.23	8.10	1.18	0.75
IL-2	10.46	0.81	[a]0.66	0.66	0.00	0.54	0.54	0.89
IL-3	0.00	0.00	0.25	0.25	0.52	0.00	0.00	0.23
IL-4	0.00	0.00	0.00	0.00	NA	0.00	0.00	NA
IL-5	0.00	0.00	[a]1.13	0.38	0.03	4.44	2.93	0.29
IL-6	0.33	0.33	[a]1100.89	253.30	0.01	*551.93	61.39	0.03
IL-9	71.66	9.38	[a]0.00	0.00	0.02	0.10	0.10	0.44
IL-10	10.28	1.00	[a]76.05	18.70	0.05	50.86	9.90	0.22
IL-12p40	13.95	1.04	[a]2.60	1.42	0.00	2.05	0.93	0.74
IL-12p70	0.43	0.43	4.40	1.64	0.15	+1.90	0.65	0.13
IL-13	26.36	9.35	[b]1.10	1.10	0.11	1.46	1.46	0.86
IL-15	25.39	2.31	17.46	3.42	0.17	13.84	2.08	0.35
IL-17	0.00	0.00	0.00	0.00	NA	0.13	0.13	0.44
IP-10	7.79	0.94	[a]8914.48	1988.21	0.01	6484.41	1667.73	0.37
KC	62.22	16.06	[a]189.71	23.66	0.01	154.54	21.81	0.31
LIF	0.00	0.00	[a]146.67	24.76	0.00	+98.20	10.55	0.06
LIX	0.00	0.00	[a]5.88	2.98	0.11	*0.61	0.55	0.05
MCP-1	0.00	0.00	[a]4542.32	789.31	0.00	*2059.69	445.21	0.01
M-CSF	0.00	0.00	[a]20.98	3.78	0.00	*9.42	1.87	0.01
MIG	12.61	2.70	[a]11497.90	645.99	0.00	22764.99	15895.91	0.58
MIP-1α	9.06	1.66	[a]70.56	7.58	0.00	55.60	6.27	0.15
MIP-1β	10.23	1.00	[a]124.30	16.73	0.00	110.07	17.77	0.59
MIP-2	50.02	4.31	[a]148.68	32.50	0.03	*62.62	5.85	0.05
RANTES	0.94	0.13	[a]12.83	1.32	0.00	*9.39	0.99	0.05
TNF-α	0.23	0.23	[a]12.14	0.53	0.00	*7.75	0.66	0.00
VEGF	16.01	1.70	[a]52.84	14.66	0.05	*22.23	2.55	0.03

SEM – standard error of the mean. NA- not applicable, *, $p<0.05$ relative to vehicle control, +, $p<0.1$ relative to vehicle control (*Eang* H2O/*Eang* EtOH compared to H2O/EtOH). [a], $p<0.05$ relative to uninfected; [b], $p<0.1$ relative to uninfected (H2O/EtOH compared to Uninfected).

Table 2. Genes upregulated by infection DAVID output.

Up Regulation Fold Change from DAVID				
Relative to Uninfected				
H2O/EtOH	*Eang*/EtOH	*Eang*/*Eang*	*Eang*/*Epara*	*Eang*/*Eang*
Day 8	Day 8	Day 8	Day 8	Day 9
	Abcf1			Abcf1
				Aimp1
	Ap3b1			Ap3b1
	C3		C3	C3
Casp1	Casp1	Casp1	Casp1	Casp1
	Cblb		Cblb	Cblb
	Ccl1	Ccl1	Ccl1	Ccl1
Ccl12	Ccl12	Ccl12	Ccl12	Ccl12
Ccl19	Ccl19	Ccl19	Ccl19	Ccl19
Ccl20	Ccl20	Ccl20		Ccl20
Ccl2	Ccl2	Ccl2	Ccl2	
	Ccl3	Ccl3		Ccl3
Ccl4	Ccl4	Ccl4	Ccl4	Ccl4
Ccl5	Ccl5	Ccl5	Ccl5	Ccl5
Ccl6	Ccl6	Ccl6	Ccl6	
Ccl7	Ccl7	Ccl7	Ccl7	
	Ccl8	Ccl8	Ccl8	Ccl8
Ccl9	Ccl9	Ccl9	Ccl9	Ccl9
	Ccnd3			
	Ccr1	Ccr1	Ccr1	Ccr1
	Ccr2			Ccr2
	Ccr3	Ccr3	Ccr3	Ccr3
Ccr5	Ccr5	Ccr5		Ccr5
	Ccr8			Ccr8
	Ccr9			
Cd1d1	Cd1d1	Cd1d1	Cd1d1	Cd1d1
Cd2	Cd2	Cd2	Cd2	Cd2
	Cd28	Cd28	Cd28	Cd28
Cd3d	Cd3d	Cd3d	Cd3d	Cd3d
Cd3e	Cd3e	Cd3e	Cd3e	Cd3e
Cd3g	Cd3g	Cd3g	Cd3g	Cd3g
	Cd4	Cd4		
Cd40	Cd40	Cd40	Cd40	Cd40
	Cd40lg			Cd40lg

Table 2. continued.

H2O/EtOH	*Eang*/EtOH	*Eang*/*Eang*	*Eang*/*Epara*	*Eang*/*Eang*
	Cd40lg			Cd40lg
		Cd74		
Cd8a	Cd8a	Cd8a	Cd8a	Cd8a
Cd8b1	Cd8b1	Cd8b1	Cd8b1	Cd8b1
Cdkn1a	Cdkn1a	Cdkn1a	Cdkn1a	Cdkn1a
	Clcf1			Clcf1
	Cxcl1	Cxcl1		Cxcl1
	Cxcl10	Cxcl10	Cxcl10	Cxcl10
Cxcl11	Cxcl11	Cxcl11	Cxcl11	Cxcl11
Cxcl12	Cxcl12		Cxcl12	Cxcl12
Cxcl13	Cxcl13	Cxcl13	Cxcl13	Cxcl13
Cxcl9	Cxcl9	Cxcl9	Cxcl9	Cxcl9
Cxcr2	Cxcr2	Cxcr2		
Dock2	Cxcr3	Cxcr3	Cxcr3	Cxcr3
	Dock2	Dock2	Dock2	Dock2
	Egr1	Egr1	Egr1	Egr1
Gadd45g	Gadd45g	Gadd45g	Gadd45g	Gadd45g
Glmn	Glmn		Glmn	Glmn
Gusb	Gusb	Gusb	Gusb	Gusb
	H2-Aa		H2-Aa	
H60a	H60a	H60a	H60a	H60a
Hells	Hells	Hells	Hells	Hells
Hprt	Hprt	Hprt		Hprt
	Icosl			Icosl
Ifng	Ifng	Ifng	Ifng	Ifng
Igbp1			Igbp1	Igbp1
Il10	Il10	Il10	Il10	Il10
	Il10ra	Il10ra		Il10ra
Il11	Il11	Il11	Il11	Il11
Il12b	Il12b	Il12b	Il12b	Il12b
	Il13ra1	Il1b		Il13ra1
	Il15		Il17b	Il15
	Il1b			Il1b
	Il1r2			
Il27	Il27	Il27	Il27	Il27
Il2ra	Il2ra	Il2ra	Il2ra	Il2ra
	Il2rb		Il2rb	Il2rb
	Il2rg		Il2rg	Il2rg
	Impdh2		Impdh2	Impdh2
	Inha			Inha

Table 2. continued.

H2O/EtOH	*Eang*/EtOH	*Eang*/*Eang*	*Eang*/*Epara*	*Eang*/*Eang*
	Itgam	Itgam		Itgam
	Itgb2	Itgb2	Itgb2	Itgb2
				Ltb
Mif	Mif	Mif	Mif	Mif
	Nos2			Nos2
	Pdcd1lg2	Pdcd1lg2		Pdcd1lg2
Pik3cd	Pik3cd	Pik3cd	Pik3cd	Pik3cd
	Prkcd			Prkcd
	Prkcq			
	Prlr			
Ptprc	Ptprc	Ptprc	Ptprc	Ptprc
Relb	Relb	Relb	Relb	Relb
	Rgs1	Rgs1	Rgs1	Rgs1
Sit1	Sit1	Sit1	Sit1	Sit1
Sla2	Sla2	Sla2	Sla2	Sla2
Spp1	Spp1	Spp1	Spp1	Spp1
				Tgfb1
Tlr1	Tlr1	Tlr1	Tlr1	Tlr1
Tlr4	Tlr4	Tlr4	Tlr4	
Tlr6	Tlr6		Tlr6	Tlr6
Tnf	Tnf	Tnf		Tnf
Tnfrsf13b	Tnfrsf13b	Tnfrsf13b	Tnfrsf13b	Tnfrsf13b
Tnfrsf1a	Tnfrsf1a	Tnfrsf1a	Tnfrsf1a	Tnfrsf1a
	Tnfrsf1b	Tnfrsf1b		Tnfrsf1b
	Tnfsf13b		Tnfsf13b	Tnfsf13b
Tnfsf14	Tnfsf14	Tnfsf14	Tnfsf14	Tnfsf14
	Traf6			Traf6
Vav1	Vav1	Vav1	Vav1	Vav1
Was	Was	Was	Was	Was
	Wwp1			

Table 3. Genes up regulated relative to vehicle control (H2O/EtOH) DAVID output.

Up regulated fold change from DAVID relative to H2O/EtOH Vehicle control			
Eang/EtOH	*Eang/Eang*	*Eang/Epara*	*Eang/Eang*
Day 8	Day 8	Day 8	day 9
			Ccr8
Ccnd3			
Cd3e			
Cd4			
Cd8a			
Cd8b1			
Cd93			
Clcf1		Clcf1	
Cr2			
Cxcr5			
Dock2		Dock2	
Gadd45g			
Hdac5		Hdac5	
Hells			
Icosl			
Il11	Il11	Il11	
Il27		Il27	
Il5ra		Il5ra	
Impdh1	Impdh1		
Inha			
Pik3cd		Pik3cd	
Prlr	Prlr	Prlr	
Ptprc		Ptprc	
Socs5			
Tlr4			
Vav1		Vav1	

Table 4. Genes down regulated by infection DAVID output.

Down Regulation Fold Change from DAVID				
Relative to Uninfected				
H2O/EtOH	*Eang*/EtOH	*Eang/Eang*	*Eang/Epara*	*Eang/Eang*
Day 8	Day 8	Day 8	Day 8	Day 9
	Ccl17	Ccl17	Ccl17	
Cd81		Cd81		
	Ccr6			
Cr2	Cr2	Cr2	Cr2	Cr2
	Cxcl15			
	Cxcr5	Cxcr5		Cxcr5
Hdac5	Hdac5	Hdac5		
				Hdac7
	Il1f8			
				Il4
Il5ra	Il5ra			
Impdh1			Impdh1	
				Jag2
Ms4a1		Ms4a1		Ms4a1
Wwp1		Wwp1	Wwp1	Wwp1

Table 5. Genes down regulated relative to vehicle control (H2O/EtOH) DAVID output.

Down regulated fold change from DAVID			
relative to H2O/EtOH Vehicle control			
Eang/EtOH	*Eang/Eang*	*Eang/Epara*	*Eang/Eang*
Day 8	Day 8	Day 8	day 9
			Cd3g
Ccl19			
Ccl20			
	Ccl3		
Ccl4	Ccl4		
	Ccl5		
	Ccl8		
Ccl9			
Crp			
Il1f8			
Tnf	Tnf		
	Tnfsf14		

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CHAPTTER IV. EFFECTS OF *ECHINACEA PURPUREA* WATER EXTRACTS AND OBESITY ON INFLUENZA INFECTED MICE

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Abstract:

Background: Obesity is associated with increased morbidity and mortality to influenza infections. Evidence suggests that dysfunction in the innate and adaptive immune responses to influenza in the obese condition may contribute to the higher rates of morbidity/mortality. *Echinacea purpurea* has been demonstrated as an immunostimulatory herbal remedy often used to treat respiratory infections. The purpose of this study was to determine the extent to which treatment with *E. purpurea* may improve immune response to influenza infection in the obese.

Methods: C57BL/6 mice were fed either a high fat diet at 60% kcal from fat (diet-induced obese mice) or a normal mouse diet for 8 weeks. One day prior to infection with Influenza A/PR/8/34 virus, mice were gavaged with either *Echinacea purpurea* water extract (ECH) treatment or water vehicle control treatment for a total of 4 groups: H2O lean, ECH lean, H2O Obese, and ECH Obese. Body weight was assessed daily (through

day 4) as a measure of illness severity. On day 3 post-infection (p.i.), bronchoalveolar lavage (BAL) and lungs were collected for analysis of viral titer, cytokines/chemokines, and cell populations. Similarly, on day 8 p.i., BAL, lungs, and spleens were collected for analysis of viral titer, cytokines/chemokines, intracellular IFN-γ production, and cell populations.

Results: In this study, there was an effect of obesity at day 3 p.i. Infected obese mice had increased IL-5, IP-10 and IFN-γ in the BAL, but decreased plasmacytoid dendritic cells (pDCs), alveolar macrophages, and CD8+ cells. The effect of ECH treatment at day 3 p.i. was a decrease in the number of conventional dendritic cells (cDC) and CD3+ cells, and reduced CD3+ cell percentage. At day 8 p.i., obese mice had greater concentrations of IL-5, but fewer alveolar macrophages, CD8+ cells, and fewer CD4+ IFNγ+ cells responding to Influenza NP peptide. The effect of ECH treatment at day 8 p.i. was increased IFN-γ by lung CD4+ and CD8+ cells in response to PMA-ionomycin and NP peptide and increased IFN-γ by spleen cells. However, the total number of BAL CD8+ cells was affected by ECH treatment differently in lean and obese mice, with a greater number of CD8+ cells in ECH treated lean mice, but a reduced number of CD8+ cells in ECH treated obese mice.

Conclusions: Overall, the obesity related effects are similar to those observed in previous studies. Although ECH did have immunomodulatory effects, ECH treatment did not reverse the obesity-associated alterations of immune response.

Introduction:

Obesity has a negative impact on immune function particularly during influenza infection. In the 2009 H1N1 pandemic, obesity emerged as a major risk factor that was associated with increased morbidity and mortality [1]. Decreased function in the cellular innate and cellular adaptive immune responses has been observed in both human and mice models of obesity [2,3]. In influenza infection of obese mice, decreases in NK cells, T cells and DC have been observed [3,4]. A therapy that could reverse the obesity-associated impairments of immunity might improve disease outcome.

There has been an increase in the use of over the counter supplements as a means to limit the severity or duration of respiratory infections including influenza. Influenza is prone to mutations and has been shown to become resistant to several anti-viral therapies, prompting some individuals to look for alternative medicine approaches [5]. *Echinacea spp.* has emerged as one of the most commonly used plant remedies for influenza infections. *Echinacea* has both immune-stimulatory and anti-inflammatory properties. Variations in preparation techniques, namely water and ethanol extractions lead to the immunomodulatory characteristics of each extract by altering the chemical profile found in each extract [6]. Water preparations are rich in polysaccharides, whereas ethanolic preparations are rich in alkamides. Caffeic acid derivatives and phenolic compounds have been shown to be in both water and ethanolic preparations [7]. *Echinacea* extracts have been shown to increase the number and function of dendritic cells (DC's), macrophages, and NK cells, while also providing anti-inflammatory effects [8-10]. The water preparations of *Echinacea purpurea* used in this study have been previously shown to exhibit immunostimulatory activity, by activating cytokine/chemokine expression in

lung epithelial cells and in murine splenocytes *in vitro* (unpublished findings). The efficacy of *Echinacea spp.* in an obese model of immune response to influenza infection has not been evaluated. With over 30% of Americans categorized as obese [11], it has become exceedingly important to evaluate the impact of obesity on the efficacy of over the counter treatments for influenza, such as *Echinacea spp*.

A murine diet induced obesity model was used to evaluate the efficacy of a water preparation of *Echinacea purpurea*. While a genetic based obesity model on C57BL/6J Ob/Ob is commercially available, these mice lack leptin signaling. Leptin signaling is important in immune function in the lung [12-14]. Also, leptin mutation is not a common cause of obesity in humans. Therefore, the diet induced obesity model may provide a more accurate modeling of human immune function in obesity. It was hypothesized that the *Echinacea purpurea* water extract (ECH) would correct the diet induced obesity immune dysfunction during influenza infection in mice. Using a high fat diet and C57BL/6J mice that are susceptible to diet induced obesity, mice were treated daily with ECH or vehicle (H2O) starting one day before intranasal infection with the mouse adapted Influenza A/PR/8/34 virus and continuing through day four post infection. Lungs, spleens, and BAL were obtained at day three and day eight post infection. These tissues were evaluated for cytokine/chemokines, cellular content or both. It was hypothesized that ECH treatment would overcome the immune dysfunction associated with diet induced obesity; however, our findings suggest that ECH does not restore the immune dysfunction associated with obesity.

Materials and Methods:

Plant & Extract.

E. purpurea (PI633669) was harvested in USDA North central regional plant introduction station at Ames, IA (USA). Water extracts were prepared by boiling 6 grams of dried root in 100 milliliters (ml) of endotoxin free water in an endotoxin free flask, and stirred for 1 hour at room temperature. Extracts were filtered using endotoxin free glass filter paper, centrifuged for 15 minutes at 10,000 revolutions per minute (rpm) and pellets discarded before freeze drying. Extracts were prepared and provided by the Hauk and Murphy labs.

Mice.

C57BL/6 mice were purchased (n=110) from The Jackson Laboratory (Bar Harbor, ME). After three days of acclimation, mice were randomly selected to receive either a high fat or low fat diet. Mice were fed either a high fat diet (Research diets 60% kcal from fat) (n=56) or normal rodent diet for 8 weeks (Research diets 10 kcal% fat) (n=54). The nutritional contents of the diet are detailed in **Table 1**. Iowa State University Committee on Animal Care approval was obtained for all procedures that were utilized in the care and experiments involved with the mice.

Gavage treatment and infection.

Animal feeding needles 20G x 1-1/2 (Cadence Science, Staunton, VA) were used to gavage the mice with either *Echinacea purpurea* (ECH) or vehicle (H2O) solutions. Extracts were diluted to 14.67 mg/ml of extract and administered at a concentration of

110 mg/kg. Mice were gavaged with ECH or H2O one day before infection and during the first 4 days of infection for a total of 6 groups: 1) n=3 untreated uninfected lean, 2) n=25 H2O lean 3) n=26 ECH lean 4) n=4 untreated uninfected obese 5.) n=26 H2O obese 6.) n=26 ECH obese. Mice were infected with 30 µl of influenza virus, A/PR/8/34, H1N1 (HA=1024, $EID_{50} = 10^{9.5}$) diluted 1:15,000 (HAU = 0.002/mouse) via an intranasal route given under isoflurane anesthesia.

Disease severity and viral load.

Mice were weighed weekly for the 8 weeks prior to infection. Mice were housed separately after infection to allow monitoring of body weight and food consumption.

Viral RNA levels were assessed by the Iowa State University Veterinary Diagnostic Laboratory using primers to detect influenza virus by fluorogenic reverse transcription polymerase chain reaction (real-time reverse transcription PCR) in the lung tissue samples submitted for titer. Primers specific to the virus conserved region of the SIV NP gene were used with TaqMan® chemistry. Sequences from GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html) and Influenza Sequence database (www.flu.lanl.gov) were utilized. A commercial vendor synthesized the forward primer (SIVRTF: 5'-CGGACGAAAAGGCAACGA-3'), reverse primer (SIVRTR: 5'-CTGCATTGTCTCCGAAGAAATAAG-3') and a TaqMan® MGB reporter probe FAM (6-carboxyfluorescein) and a non-fluorescent quencher (SIVRTP: 5'-6FAM-CCGATCGTGCCYTC). Primers were synthesized by Integrated DNA Technologies (Coralville, IA), and the probes were synthesized by Applied Biosystems (Foster City, CA). Viral RNA was extracted from 50µl of lung tissue using the Ambion®

MagMAX™ Viral RNA Isolation kit (Applied Biosystems) and a KingFisher® 96 magnetic particle processor (Thermo Scientific, Waltham, MA). H1N1 and H3N2 swine influenza viruses and elution buffer were used as positive and negative controls, respectively. The QuantiTect® Probe RT-PCR Kit by Qiagen (Valencia, CA) was used to perform the real time reverse transcription-PCR. Each reaction consists of 4 µl of lung sample with 0.4 µM (final concentration) of each primer and 0.2 µM (final concentration) of the probe for a total volume of 20 µl for each reaction. Using a 384 well format and the ABI 7900HT Sequence Detection System (Applied Biosystems), reverse transcription occurred for 30 minutes at 50°C. Immediately following reverse transcription, the RT-PCR was activated with 15 minutes at 95°C. Activation was followed by 40 cycles of 15 seconds at 94°C and 60 seconds at 60°C. To generate a standard curve, a set of influenza preparations with known concentrations of viral titers (EID50/ml) were used. Each sample then could be extrapolated by using the standard curve to convert the threshold cycle (Ct) value to a viral titer [15].

Tissue Processing.

Lung and spleen were harvested in stomacher bags containing 5 ml of digestion media (RPMI with 2% fetal bovine serum (FBS) with collagenase D at a concentration of 1 mg/ml). Lung and spleen tissues were subjected to Seward Stomacher® (Port St. Lucie, FL) paddle blender. Lung and spleen were processed through 0.4µ cell filters with digestion media. The plunger of a syringe was used to break up the remaining tissue through the filter, and the filtrate was suspended in 10 ml of digestion media. Samples were centrifuged for 10 minutes at 1200 RPM, washed twice with complete RPMI (cRPMI), and re-suspended in 5 ml of cRPMI (RPMI with Hepes, glutamate, sodium

pyruvate, penicillin/streptomycin, fungizone, 2-mercaptoethanol and 10% FBS) without collagenase D. BAL cells were harvested by centrifugation at 1200 RPM for 10 minutes and re-suspended in 250 µl of, BD stain buffer (PBS- azide+0.1%BSA)(BD Biosciences). All samples were treated with 2 mls of ice cold 0.015M ammonium chloride red blood cell lysing agent for 10 minutes, washed in cRPMI (lungs, spleen, lymph nodes) or BSA (BAL cells) and re-suspended in 5 ml of cRPMI or 250µl BD stain buffer . Cell counts were obtained using the Z™ series Coulter Counter® (Beckman-Coulter, Inc., Brea, CA).

Flow Cytometry.

Cell count for each sample was determined and adjusted to a range from $1x10^5$ and $1x10^6$ per ml. Samples less than $1x10^5$ cells per ml were combined with another sample from the same treatment group (BAL group only). Adjusted cell samples (250 µl) were transferred to a 96 well round bottom plate for testing. Intracellular cytokine assessment was performed on the lung cells only, not on cells from the BAL. For flow cytometry, each well was treated with 10 µl of Fc block 1:10 dilution (0.5 mg/ml) (BD Biosciences) for 10 minutes. Day 3 BAL cells were incubated with the following antibodies: PE-anti-mouse Ly6G, APC-anti-mouse mPDCA, PE-Cy7 anti-mouse CD11c, PerCp-Cy5.5-anti-mouse CD3e, AF647 anti-mouse CD11b, FITC- anti-mouse Ly6C, and APC-Cy7- anti-mouse CD8a. Cell populations were defined by the following markers (**Table 2**): Total live cells (live gate), conventional dendritic cells (cDC) (Autofluor-, CD11b+CD11c+LyC+LyG+), plasmacytoid dendritic cells (pDC) (Autofluor-, CD11c+, LyC+, LyG-, mPDCA+), alveolar macrophages ($CD11b^{lo}$, CD11c+, Ly6C-, Ly6G-, Autofluor+), polymorphonuclear cells (PMN)- neutrophil, Autofluor-, CD11b+, CD11c-, LyC+, LyG+, SSC+), monocytes (Mono) (Autofluor-, CD11b+, CD11c-, LyC+, LyG+,

SSA-), CD8a (Autofluor-, CD11b-, CD11c-, CD3e+, CD8a+), and CD3e+ (Autofluor-, CD11b-, CD11c-, CD3e+, CD8a-). Day 8 BAL cells were treated with PE- anti-mouse Gr1, APC-Cy7-anti-mouse CD8a, PE-Cy7-anti-mouse CD11c, AF 647 anti-mouse CD11b, AF700 anti-mouse MHCII, PE-Cy5 anti-mouse CD4, and PerCp-Cy5.5-anti-mouse CD3e. Cell populations were defined by the following: alveolar macrophages (CD11b+CD11c+Gr1-Autofluor+), conventional dendritic cells (cDC) (Autofluor-, $CD11b^{lo}$, $CD11c^{int}$, $MHCII^{int}$), inflammatory monocytes (iMono) (Autofluor-, CD11b+, CD11c-,Gr1+, SSC-), PMN (neutrophil) (Autofluor-, CD11b+, CD11c-,Gr1+, SSC+), CD8a+ (Autofluor-, CD11c-,Gr1-, CD3e+, CD8a+), CD4+ (Autofluor-, CD11c-, Gr1-, CD3e+, CD8a-, CD4+), cross-presenting CD8+ dendritic cells (xDC) (Autofluor-, $CD11b^{lo}$, $CD11c^{int}$, CD3e-,CD8a+). Day 8 spleen cells were treated AF488 anti-mouse CD8b, PE-Cy7 anti-mouse CD11c, APC-Cy7 anti-mouse CD19, and Pe-Cy5 anti-mouse CD4. Cell populations were defined by the following: CD4 T-cells (CD11c-, CD4+, CD8b-), CD8b T-cells (CD11c-, CD4-, CD8b+), B cells (CD11c-, CD4-, CD8b-, CD19+). Samples were mixed and incubated for 30 min. at 4°C protected from light (wrapped in aluminum foil). After incubation, ~150 µl of BSA was added to each sample. The plates were centrifuged at 1200 RPM for 5 minutes at 2-4°C. Supernatant was removed by pipetting and 2 more wash steps were performed using 250 µl of BD Stain buffer for each wash. After the final wash step, supernatants were removed, cells were gently mixed by placing the plate on a vortex, and 150 µl of diluted BD™ stabilizing fixative was added to each sample. Samples were then transferred to 5 ml BD Falcon™ tubes and stored at 2-4°C covered in foil (protected from light) until analyzed

on a BD FACSCanto™ Flow Cytometer (BD, Franklin Lakes, New Jersey) by Iowa State Flow Cytometry Facility.

Intracellular flow cytometry staining for intracellular cytokines.

Using a 96-well V bottom plate, 200 µl of lung cells ($1x10^6$/ml) were added to four wells for each sample. Controls for intracellular flow were plated by combining several samples and plating four wells for each color to be used. Samples were stimulated with one of the following in one well for each sample: 10 µl of 1 µM of NP peptide, $NP_{366-374}$ (ASNEMNDAM),), 5 µl of 1:10 diluted Leukocyte Activation Cocktail, with BD GolgiPlug™ (LAC) (BD Biosciences) in cRPMI (positive control containing PMA/Ionomycin), or 10 µl of cRPMI (unstimulated control). The protein transport inhibitor, BD GolgiPlug™ (containing Brefeldin), was added 2.5 µl of 1:10 dilution to each well except the LAC which already contained the BD GolgiPlug™. Samples were placed in a humidified incubator at 37°C with 5% CO2 for 6 hours. Samples were centrifuged at 1600 RPM for 10 minutes at 4°C. Supernatants were removed by gentle pipetting, and 100 µl of BSA was added to each well. Each well was treated with 10 µl of Fc block 1:10 dilution (0.5 mg/ml) for 10 minutes. Extracellular antibodies were added, 5 µl of 1:100 dilution of PE Cy-5 anti-mouse CD4 (0.25 µg/test) and 12.5µl of 1:100 dilution of AF-488 anti-mouse CD8b (0.25 µg/test). Plates were incubated for 30 minutes at 4°C and protected from light with foil. Samples were centrifuged at 1600 RPM for 5 minutes at 4°C and washed twice with 200 µl of BD Stain Buffer. After the last wash, samples were re-suspended in 100 µl of Fixation/Permeabilization solution (BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit, BD Biosciences, San Jose, California). After incubation with Fix/Perm for

20 minutes at 4°C protected from light, samples were washed with 1X BD Perm/Wash™ Buffer BD (250 µl/wash/well) and re-suspended in 100 µl of BD Perm/Wash™ Buffer (BD Perm/Wash™ BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit, San Jose, California). Intracellular antibodies were added: 5 µl of 1:100 dilution PE anti-mouse IFN-γ (0.25 µg/test) and 5 µl of 1:100 dilution Alexa Fluor 700 anti-mouse TNF-α (0.25 µg/test). Samples were incubated at 4°C for 30 minutes protected from light. After incubation, samples were washed twice with 1X BD Perm/Wash™ Buffer BD (250 µl/wash/well) and re-suspended in 150 µl of 1X BD™ stabilizing fixative. Samples were transferred to 5 ml BD Falcon™ tubes and stored at 2-4°C covered protected from light until analyzed on a BD FACSCanto™ Flow Cytometer (BD, Franklin Lakes, New Jersey) by Iowa State Flow Cytometry Facility.

Spleen Culture.

Spleens were processed as described above. Cells were re-suspended in cRPMI at $1x10^6$cells/ml, and cultured into 96 well plates (200 µl per well, 6 wells per spleen). Three wells for each spleen were stimulated with 10 µl of A/PR/8/34, H1N1 (HA=1024, $EID_{50} = 10^{9.5}$) diluted to 100 HAU/$1x10^6$ cells (UV inactivated for 30 minutes), while the other three wells received 10 µl of media alone for control. Final volume for each well was 300 µl. Samples were incubated at 37°C in a 5%CO2 humidified incubator, and supernatants were collected at 48, 72, and 96 hours post stimulation. Supernatants were frozen at -20°C until further analysis.

ELISA.

BD OptEIA™ Mouse IFN-γ (BD Biosciences, San Diego, CA) was used to analyze the supernatants from the spleen culture for IFN-γ. A 96-well plate was coated with 100 µl per well of diluted Capture Antibody (1:250 in Coating Buffer prepared per package instructions). The plate was incubated overnight at 4°C. Using wash buffer (PBS plus 0.05% Tween-20), the plate was washed 5 times with 300 µl and blotted. Plates were blocked with 200 µl Assay Diluent for 1 hour at room temp and washed again 5 times with 300 µl of wash buffer. Standards were diluted using a 2-fold serial dilution from 2000 pg/ml to 31.3 pg/ml. Samples were tested in undiluted state. Each standard or sample tested was added to each well (100 µl) and incubated at room temp for 2 hours. After washing 5 times with 300 µl of wash buffer, 100 µl of working detector was added (Detection antibody, diluted 1:250 in assay diluent plus SAv-HRP reagent diluted 1:250 in detection antibody). Samples were incubated for 1 hour at room temp with working detector before washing the plate 10 times with 300 µl of wash buffer (allowing the wash to sit for 1 minute before removing). Substrate solution (equal amounts of Tetramethylbenzidine [TMB] and Hydrogen Peroxide, BD Pharmingen™TMB Substrate Reagent Set) was added to each well (100µl each) and incubated in the dark for 30 minutes at room temperature. After which, stop solution (2N H2SO4) was added to each well (50 µl). Plate was read at 450 nm on BMG FLUOstar Galaxy.

Multiplex Assay.

Bronchoalveolar lavage fluid (BAL) was collected to measure cytokine/chemokine concentration. The BAL was separated into supernatant and cellular portions by centrifugation at 1200 RPM for 10 minutes. The Milliplex® microsphere (EMD Millipore Corporation, Billerica, MA) assay was used to analyze BAL supernatant for expression of 19 different cytokines and chemokines that could be present in the lung of the infected mice. The preparation of the assay was performed as package instructions indicated utilizing the overnight incubation of 18 hours. The assay data was collected using Bio-plex® Suspension Array System (Bio-Rad laboratories, Inc, Hercules, CA) instrumentation. Data was exported from Bio-plex® Manager to Microsoft® Excel (Microsoft, Redmond, WA) for further analysis.

Analysis.

Statistical analysis was performed with a 2-way ANOVA (obesity * *Echinacea* treatment) using SPSS® software version 20 (IBM Corporation, Armonk, New York). For body weight analysis, a repeated measures ANOVA was used. If a treatment by time interaction was observed, follow-up analyses evaluated the effect of *Echinacea* separately for lean and obese mice. If an interaction of obesity and ECH was observed follow up analysis were performed using one-way ANOVA. One-way ANOVA analysis was also used to evaluate the uninfected control mice to the ECH and H2O treatment groups for each diet.

Results:

It was hypothesized that ECH treatment of obese mice would stimulate the immune system and overcome the obesity associated immune dysfunctions. The first measure to test this hypothesis was to evaluate clinical evidence of illness severity. This was measured using daily weights and lung viral titer. With respect to the mice that were euthanized on day 3 post-infection, a significant interaction between time and obesity status was observed ($p<0.05$). In follow up analyses, the daily weight loss in grams was not statistically different in the ECH treatment in obese or lean mice compared to mice treated with H2O (Figure 1a). In the mice euthanized at day 8 post-infection, there was a trend ($p=0.06$) for a time by obesity by ECH treatment interaction, such that obese mice treated with ECH tended to lose less weight (Figure 1b). However, when weight loss was expressed as percent of original body weight, there was not an effect of ECH treatment on weight loss in either the obese or lean mice. Food consumption was also monitored over the infection period (Figure 2). Infected mice consumed less total kcal over the infection period as expected, although there were no significant differences between the ECH and VEH control groups.

In this particular study, there was a higher mortality than expected due to the stress sensitivity of the C57/BL6 not directly a result of the infection. There appeared to be mortality associated with the stress of gavage treatment. ECH dosing was discontinued on day 4, after which the mortality returned to normal levels. Viral RNA level analysis was conducted using whole lung lobes. Although there was no statistically significant difference in viral RNA levels at day 3 p.i. or day 8 p.i., there was a trend towards reduced viral load in ECH-treated obese mice by day 8 (Figure 3).

To further investigate the effect of ECH treatment on immune response in lean and obese mice, we analyzed cytokines and chemokines in BAL fluid collected from the mice on day 3 p.i. and day 8 p.i. (Figure 4 and Table 3). On day 3 p.i., there was a main effect of obesity for IL-5, IP-10, and IFN-γ such that obese mice had significantly greater levels present in the BAL compared to the BAL from lean mice. Cytokines and chemokines that were analyzed for each treatment group with are shown in Table 3.

Cellular response in the lung BAL fluid was also evaluated at day 3 p.i. Although diet induced obese mice receiving ECH treatment did not have altered total cell number in the BAL compared to the lean mice, there were changes in cell subpopulations (Figure 6 and Table 4). Obese mice had significantly fewer plasmacytoid dendritic cells (pDC) (both counts and percentages). ECH treatment resulted in fewer CD3+ cells, both total number and percentage. ECH also tended to be associated with decreased numbers of conventional dendritic cells (cDC) count and percentage. These effects of ECH treatment were demonstrated in both lean and obese mice.

On day 8 p.i., BAL fluid was also analyzed for cytokines/chemokines (Table 3), total lung cell count (Figure 5) and cell populations (Figure 7 and Table 5). Similar to day 3, obese mice tended to have higher concentrations of IL-5 in the BAL. There were no significant effects of ECH treatment on cytokines or chemokines in the BAL at day 8. At day 8 p.i., cell populations were altered in the obese condition and as a result of ECH treatment. With respect to obesity, there were reduced percentages of alveolar macrophages, CD8+ cells, and a trend toward fewer CD4+ cells in obese mice relative to non-obese mice. The effect of ECH treatment was different in lean mice compared to obese mice. In lean mice, ECH treated mice had a greater percentage of CD8+ cells

whereas in obese mice, ECH treated mice had reduced percentage of CD8+ cells (significant interaction for CD8 at $p < 0.05$; with a trend towards a similar effect in CD3+ cells at $p = 0.09$).

The lung tissue from mice was used to assess total cell count, alveolar macrophages, CD4+ cells, and CD8+ cells. Overall, ECH tended to reduce the total number of cells in the lung, although this effect only approached statistical significance (Figure 8). There were no significant effects of ECH treatment on alveolar macrophages, although a trend was observed such that the total number of macrophages declined in lean mice, but the actual percentage of alveolar macrophages tended to increase only in obese ECH-treated mice (diet * ECH interaction, $p < 0.10$). ECH treatment also reduced overall lung CD8%, and tended to decrease CD8 cell number. Mice in the obese condition had reduced overall cell count, reduced alveolar macrophage, as well as a decrease in the percentage of CD8+ cells (Figure 8).

Also, the number and percentage of CD4+ IFN-γ+ and CD8+IFNγ+ cells were determined under each *in vitro* condition (Figure 9). Obese mice treated with ECH had a greater percentage of CD4+ IFN-γ+ cells responding to influenza NP peptide, but this effect was not observed in lean mice (d * e interaction). However, both obese and lean mice treated with ECH had an increase in the percentage of CD8+ IFN-γ+ NP-responding cells. When PMA-ionomycin was used to activate IFN-γ as a non-specific stimulator, ECH treated mice had a greater percentage of CD8+ cells producing IFN-γ ($p < 0.05$). These results suggest that ECH may act as a non-specific activator, and obesity may be associated with a reduced influenza-specific response (Figure 9).

Lastly, the spleen cell populations were evaluated by flow cytometry for cell populations and by ELISA for IFN-γ production at day 8 p.i. Total spleen cell counts (Figure 10), total spleen CD8 cell, CD4+ cell and CD19+ B cell counts (data not shown) were not different between lean and obese mice and did not change in response to treatment with ECH. With respect to cell percentages, the actual percentage of CD8+ cells was higher in ECH obese mice compared to obese mice treated with vehicle, but the percentage of CD8+ cells in lean mice did not change with ECH treatment (significant interaction, $p < 0.05$) (Figure 10). IFN-γ production was analyzed in spleen cultures stimulated with UV inactivated A/PR/8/34 at 48, 72, and 96 hours post-stimulation (Figure 11). There were significant interactions between time and obesity as well as between ECH treatment and time ($p < 0.05$) at 72 and 96 hours. A significant diet * ECH interaction was observed in the 48 and 96 hour IFN-γ production. At 48 hours, treatment with ECH tended to decrease IFN-γ production in lean mice ($p=0.08$), while no change was observed in the obese treatment group. ECH treatment increased IFN-γ production at 72 hours post stimulation but declined by 96 hours. It is therefore possible that ECH treatment altered the kinetics of cytokine production. At the 96 hour time point, the IFN-γ level in obese was lower than lean mice ($p < 0.05$). It is possible that the peak levels of IFN-γ peak at an earlier time point in obese mice. A main effect of diet was observed at 48 hours and 96 hours ($p<0.05$). At both 48 and 96 hours obese mice had less IFN-γ production in the spleen cells relative to the lean mice.

Discussion:

Diet induced obesity results in dysfunction of both the innate and adaptive immune responses to influenza. *Echinacea spp.* has been shown to enhance the innate

and adaptive immune responses in mice feed normal mouse diet, but the effect on diet induced obesity is unknown. It was hypothesized that ECH treatment of obese mice would restore the obesity associated immune dysfunctions. The only immune parameters measured in which ECH treatment appeared to improve response among obese mice was an increase in alveolar macrophage percentage, CD4+IFN γ+% and CD8+IFNγ+% cells in the whole lung when stimulated with NP-peptide and PMA-ionomycin. There were decreased numbers of total lung cells and CD8 cells and percentage in the whole lung of diet induced obese mice treated with ECH. This decrease in cellular infiltration could be a result of infection resolution with the decrease in viral titer in ECH treated mice at day 8. There was also an effect of ECH on the kinetics of IFN-γ production in stimulated spleen cells. It was hypothesized that ECH would correct the obesity associated cellular immune function. While there were no significant changes in the obese mice CD8 count when treated with ECH, there were increases in CD4+IFN-γ+% and CD8+IFN-γ+% cells in ECH treated mice. ECH also appears to be a non-specific stimulator of the immune response that could provide benefit in preventing influenza associated secondary infections. *Echinacea* has been shown to enhance the innate and adaptive immune responses in mice feed normal mouse diet. It has been shown to be both stimulatory and anti-inflammatory through modulation of the expression of cytokines and chemokines and increases cellular immune activity [9,10,16]. There was also an effect of ECH on day 3 BAL with decreases in cDCs and CD3e+ counts and percentages. Circulating CD8 and CD4 T cells are decreased in obese patients [17]. With decreased numbers in circulation, decreased cells in circulation could translate into delay in the response to infection.

Obesity related changes at day 3 post-infection were increased of IL-5 and IP-10, and a tendency towards increased IFN-γ in the BAL, along with decreased pDCs. By day 8 p.i., the BAL fluid of obese mice still had a higher concentration of IL-5, but there were decreases in the percentage of alveolar macrophages and CD8+ cells in the BAL. In the cells from BAL at day 3, there was an obesity effect on pDC cell count and percentage, and day 8 there were decreases in alveolar macrophage percentage and CD8. Decreased numbers of pDC would be associated with an impaired immune response to influenza. These cells produce the anti-viral type I IFN. Decreases in alveolar macrophages have been shown to regulate in cytotoxic CD8 function [18]. Reductions in all three of these cell types at day 3 may correlate with the delayed response to influenza that has been associated with the obese [2]. Another study demonstrated a decrease in IFN-α/β, TNFα, IL-6, and IL-10 the lungs of mice at day 3 p.i. [3]. This correlates with the decreased cell populations in our study with decreased pDC, alveolar macrophages, and CD8 which would be involved in producing those cytokines. It has also been shown that the obesity condition alters the antigen presentation function of DCs and results in reduced antigen specific CD8 cells recruited to the lung [4]. These findings would lead to decreased CD8+ and CD4 cells in the lungs of diet induced obese mice, a finding we observed in this study with decreased CD8+ in the BAL at day at as well as decreased whole lung cell totals, CD8 cell number, alveolar macrophage percentages, and CD4+IFNγ+% cells.

With the NP stimulation of the lung cells from obese mice, there was a decrease in both the CD8+IFNγ+ and CD4+IFNγ+ cells compared to the lean mice suggesting an influenza-specific immune impairment. It has been shown that CD8+ cells from obese mice do not adequately produce IFN-γ in response to secondary challenge with influenza

[19]. CD4+IFNγ+ cells in the lung have been associated with improved clearance and protection from lethal influenza infection, with these CD4+ cells having an effecter function in the lung, producing IL-10 and IFN-γ [20]. While decreased CD4 response in primary infection has associated with a decrease in the CD8 response on secondary challenge [21]. In this study, our findings correlate with the influenza-specific immune impairment with CD8+ cells producing less IFN-γ compared to lean mice when stimulated with influenza NP peptide. ECH treatment could possibly provide improvement to the obesity impairment as seen with the ECH treated groups demonstrating an increase in CD8+IFNγ+ cells and CD4+IFNγ+% when stimulated with PMA-ionomycin. This suggests that ECH is a non-specific activator of CD4 and CD8 in the lung.

In this study there were several interactions with ECH treatment and diet such that the ECH treatment resulted in different outcomes depending on the obesity condition. There was an interaction with respect to the percentage of macrophages in the lungs. ECH treated lean mice had fewer macrophages compared to the obese ECH treated mice. Both lean and obese mice had lower total lung cell counts with ECH treatment with a higher percentage of alveolar macrophages present in the obese ECH treated mice. This could provide a benefit to the obese mice by promoting viral clearance.

In the spleen, the percent CD8+ cells was increased with ECH treated obese mice compared with the ECH treated lean mice. This could be a result in the increased viral clearance and correlated with the trend in the decreased viral titer seen in the ECH treated obese mice. IFN-γ production in the stimulated spleen immune cells was increased with ECH treatment at 48 and 72 hours and obese mice had decreased IFN-γ production

compared to the lean at 96 hours suggesting that ECH treatment altered the kinetics of cytokine production in the obese mice. This could also be a result of increased CD8+ cells in the spleen.

Endotoxin activity in botanical treatments remains in question. One limitation of this study is the presence of endotoxin in root preparations of *Echinacea.* It has been suggested that the macrophage activation seen in the *in vitro* studies with *Echinacea* treatment is a direct response from the endotoxin levels in the extract [22,23]. Although this remains unconfirmed *in vivo*, the non-specific activation of IFN-γ production in the CD4 and CD8 cells in response to LAC treatment in the intracellular staining experiment in this study could be a result of presence of endotoxin. Follow up analysis of endotoxin concentration would be necessary to confirm presence of endotoxin.

In this study, we utilized a short-term diet induced obesity model with the mice consuming a high fat diet for 8 weeks. DEXA analysis was performed previously in our lab to establish the adiposity of mice after this duration of high fat diet (unpublished data). The DEXA analysis was not re-evaluated in this study, but the results from the previous study was used to determine a proper duration of the diet for DIO conditions. Because of the short duration of the obesity condition of the mice in this study, the results from this experiment would not necessarily reflect the leptin insensitivity observed in the long term obese condition. Leptin insensitivity could potentially be investigated with *Echinacea* using a high fat diet given over a longer duration [24]. This study also does not address the impact of genetic (leptin deficiency) development of obesity as mentioned with the design and selection of the DIO model of obesity, nor does it address the prospect of insulin resistance. In addition, further studies should evaluate the potential

of enhanced immunological memory response with ECH treatment in diet induced obese mice as well as susceptibility of ECH treated, influenza infected mice to secondary bacterial infections, a common cause of influenza associated mortality in both lean and obese humans.

Taken together, the obesity effects seen in the diet induced obesity mice used in this study correlated with the findings of previous studies. To the best of our knowledge, no previous studies have evaluated the effect of ECH treatment in a diet induced obesity model of influenza infection. ECH treatment increased percentage of alveolar macrophages while decreasing CD8 counts in the lungs of obese mice, and also altered the IFN-γ production in spleen cells. Overall, ECH treatment did not correct the obesity associated immune changes seen in diet induced obesity, but could provide a benefit in increased viral clearance and faster recovery through increased alveolar macrophages and CD8+IFNγ+%, and CD4+IFNγ+% cells in the lung.

Figures for Chapter IV.

Figure 1a.

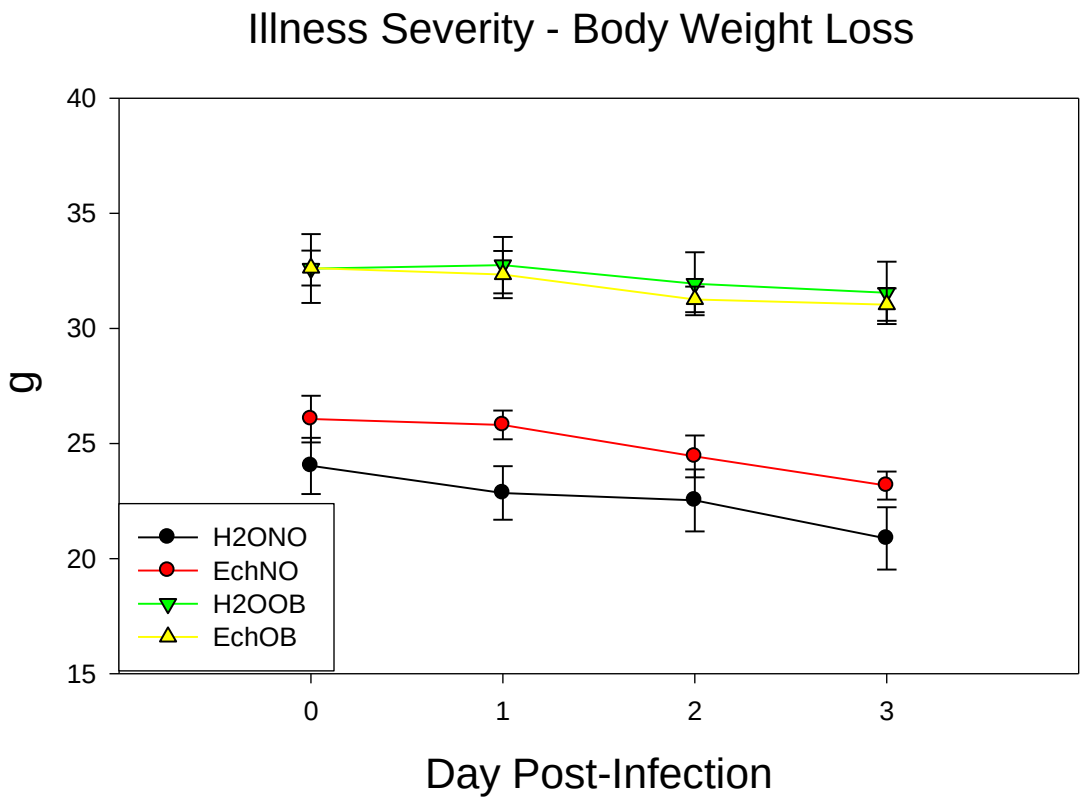


Figure 1b.

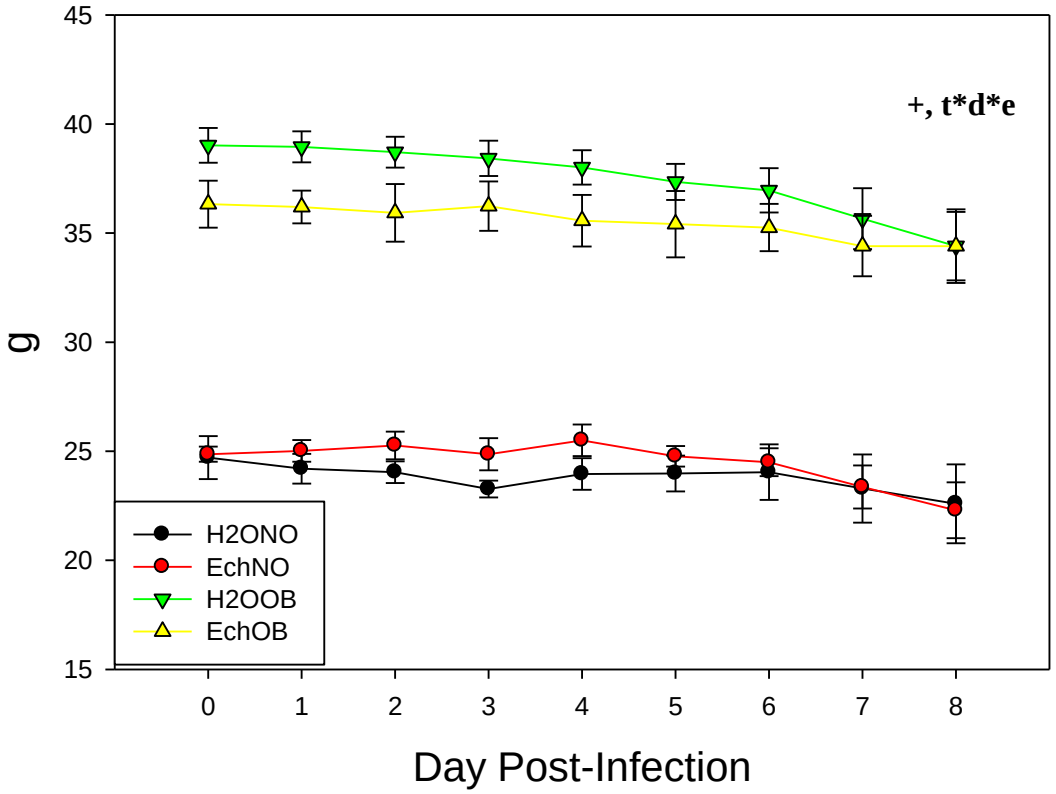


Figure 1a, b. Daily weights of mice infected with A/PR/8/34. a.) Day 3 b.) Day 8. No significant changes weight loss rates were observed in the treatment at day 3 or day 8. Each day represents the mean +/- SEM. Time*obesity*extract interaction. Obese mice treated with ECH tended to lose less weight over time. +, t*d*e, p=0.06

Figure 2.

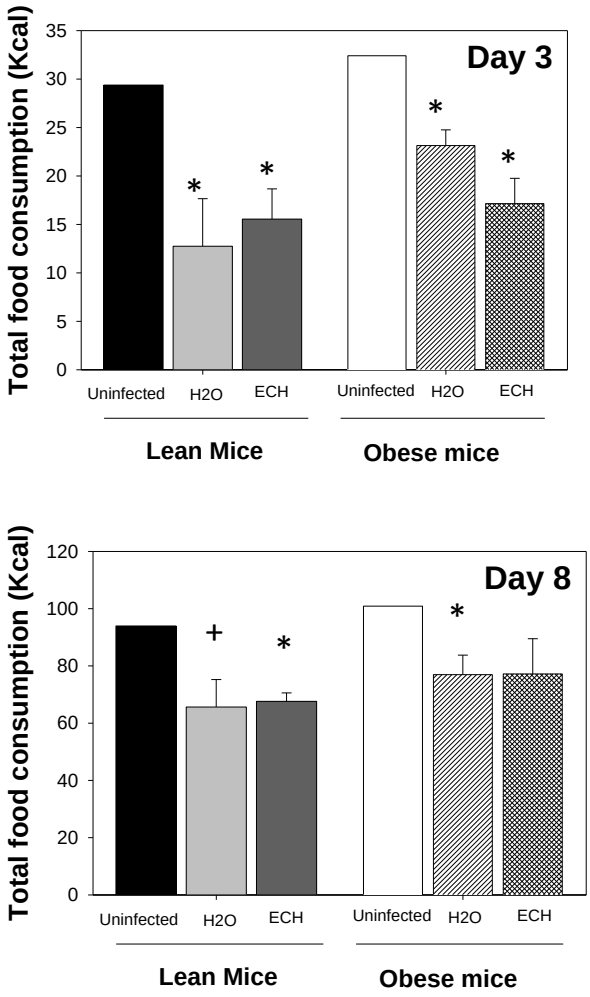


Figure 2. Total food consumption post infection in kilocalories (kcal).

Total food consumption of each group of mice over either the 3 day or 8 day experiment post infection. Values are given in mean +/-SEM. Grams were converted to energy relevant to each diet consumed in total kcal. Infected mice consumed less food during the infection period compared to the uninfected mice during the same time frame. *,

p<0.05 +, p=0.06. Uninfected Lean (day 3: n=3; day 8: n=3), Uninfected obese (day 3: n=4; day 8: n=4), H2O lean (day 3: n=4; day 8: n=4), ECH lean (day3: n=6; day 8: n=5).

Figure 3.

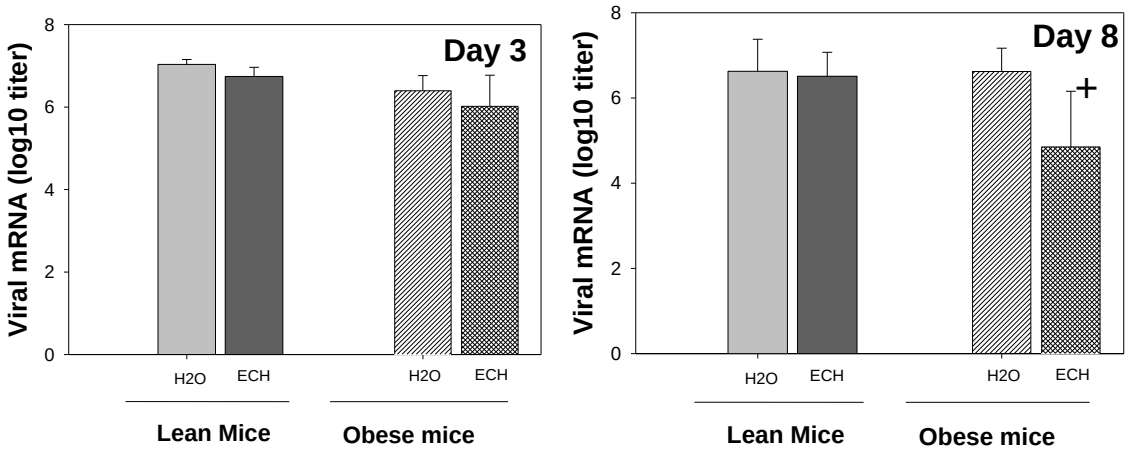
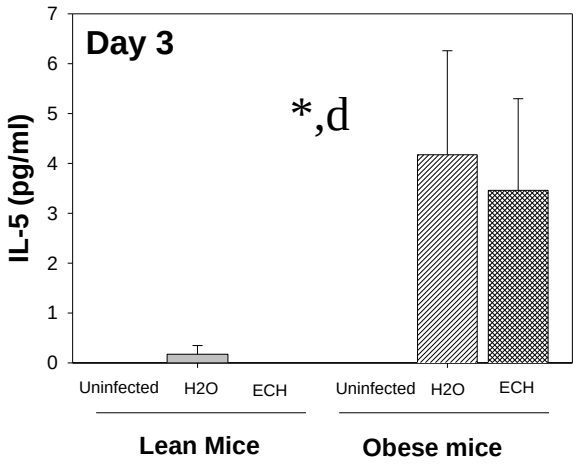
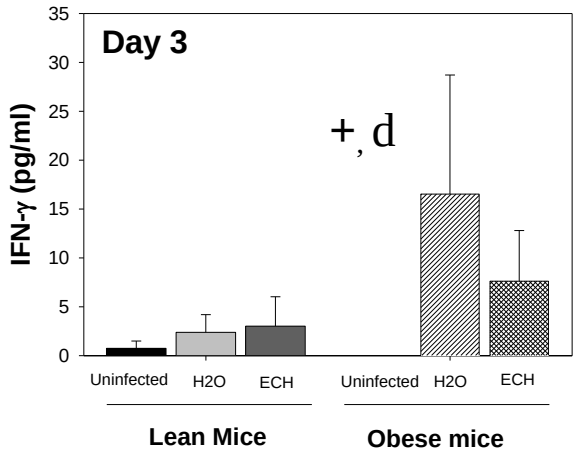


Figure 3. **Lung Viral titer in mice Day 3 and Day 8 post infection (p.i.) with influenza A/PR/8/34 virus and treatment with *Echinacea* or vehicle control.** Log 10 of the mean viral load +/- SEM from lungs obtained on day 3 p.i. and day 8 p.i. There was no difference in viral load between treatment groups at day 3 p.i. However, at day 8 p.i., treatment with ECH tended to decrease viral load in the lungs of obese mice on day 8 p.i. Uninfected n=3; **day 3**: H2O lean, n=3 ECH Lean n=4; H2O obese n=3; ECH obese n=3; **day 8**: H2O lean, n=4; ECH Lean n=5; H2O obese n=7; ECH obese n=3. **+,** p=0.10.

Figure 4.


Day 3
*,d
IL-5 (pg/ml)
0
1
2
3
4
5
6
7
Uninfected
H2O
ECH
Uninfected
H2O
ECH
Lean Mice
Obese mice


Day 3
+, d
IFN-γ (pg/ml)
0
5
10
15
20
25
30
35
Uninfected
H2O
ECH
Uninfected
H2O
ECH
Lean Mice
Obese mice

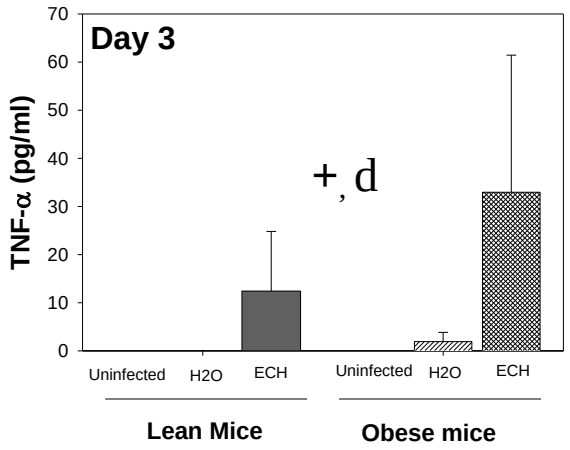

Day 3
+, d
TNF-α (pg/ml)
0
10
20
30
40
50
60
70
Uninfected
H2O
ECH
Uninfected
H2O
ECH
Lean Mice
Obese mice

Figure 4. continued.

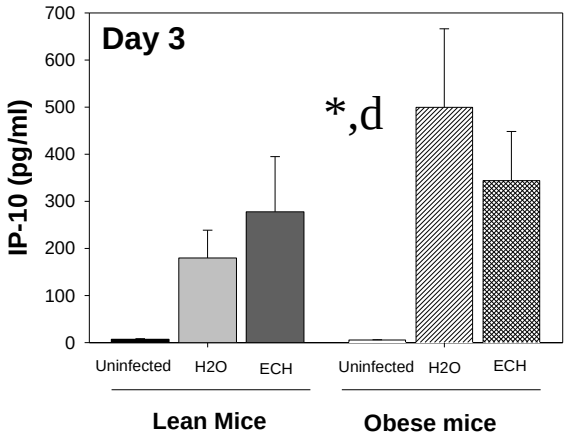


Figure 4. Day 3 Main effect of the obesity condition on BAL cytokines and chemokines. BAL collected from mice infected with influenza and treated with ECH or H2O at day 3 p.i. BAL supernatants were collect and analyzed for cytokines/chemokines by Bio-Plex multiplex array. The main effect of obesity showed an increase in IL-5 ($p<0.05$), IP-10 ($p<0.05$), and had a trend towards an increase in TNF-α and IFN-γ ($p=0.07$) compared to lean mice. Each graph represents the mean +/- standard error. Uninfected n=3; H2O Lean n=3; ECH Lean n=4; H2O Obese n=3; ECH Obese n=3. *, d $p<0.05$ main effect of obesity; +, d $p=0.1$ main effect of obesity using an ANOVA analysis.

Figure 5.

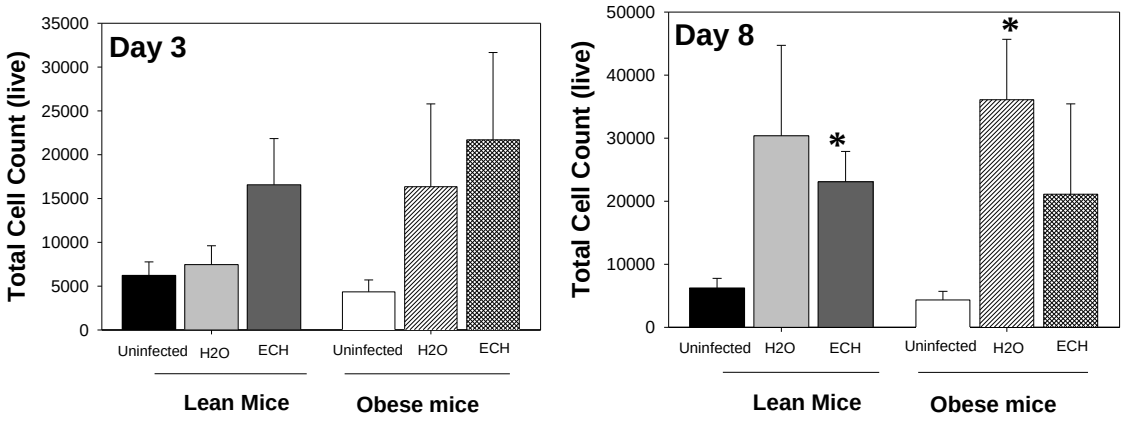


Figure 5. BAL total cell counts from Day 3 and Day 8 post infection with A/PR/8/34 and treatment with either *Echinacea* or Vehicle Control. BAL was collected day 3 p.i. and day 8 p.i. and analyzed using flow cytometry. Total cell counts were not statistically significant due to the high variation in counts. Day 8 counts were significantly different from uninfected mice by ANOVA. *, $p<0.05$. Data shown are mean values +/- SEM. Uninfected n=3; **day 3**: H2O lean, n=3 ECH Lean n=4; H2O obese n=3; ECH obese n=3; **day 8**: H2O lean, n=4; ECH Lean n=5; H2O obese n=7; ECH obese n=3.

Figure 6.

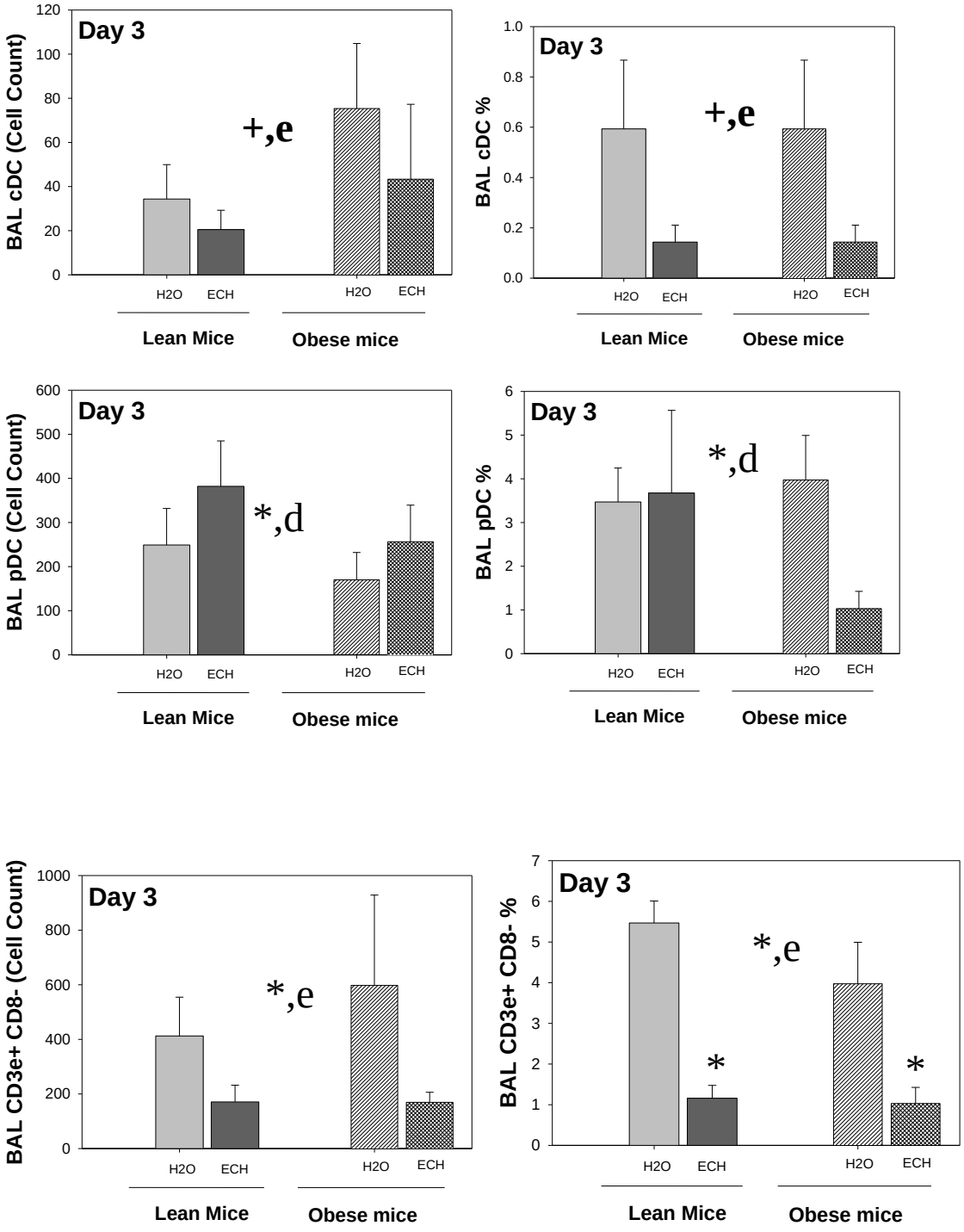

Day 3
BAL cDC (Cell Count)
+,e
H2O
ECH
Lean Mice
Obese mice
Day 3
BAL cDC %
+,e
Day 3
BAL pDC (Cell Count)
*,d
Day 3
BAL pDC %
*,d
Day 3
BAL CD3e+ CD8- (Cell Count)
*,e
Day 3
BAL CD3e+ CD8- %
*,e
*
*

Figure 6. BAL Cell populations from Day 3 post infection. Mice were infected with A/PR/8/34 and treated with either ECH or H2O control. BAL was collected 3 days p.i. and analyzed for cell populations using flow cytometry. Mean cell counts for each population+/- SEM and % of live cells +/- SEM. A main effect of obesity was observed with decreased pDC count ($p<0.05$). There was a main effect of ECH treatment with decreased cDC cell count ($p=0.07$) and percent (0.06); decreased CD3+CD8- cells count and percent ($p<0.05$). *, $p<0.05$; +, $p=0.10$, *, e ECH effect <0.05; *, d Obesity effect $p<0.05$. +, e ECH $p<0.10$ H2O lean, n=3 ECH Lean n=4; H2O obese n=3; ECH obese n=3.

Figure 7.

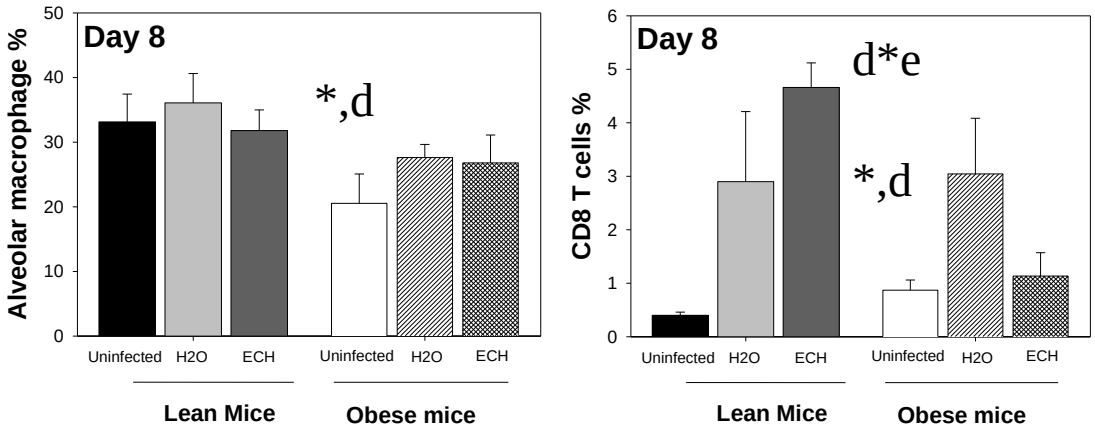


Figure 7. continued.

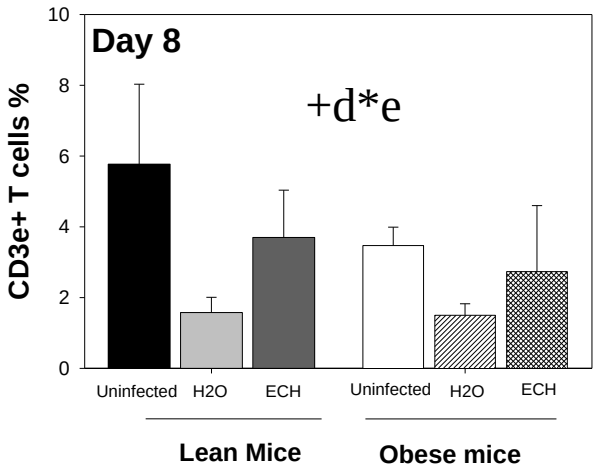
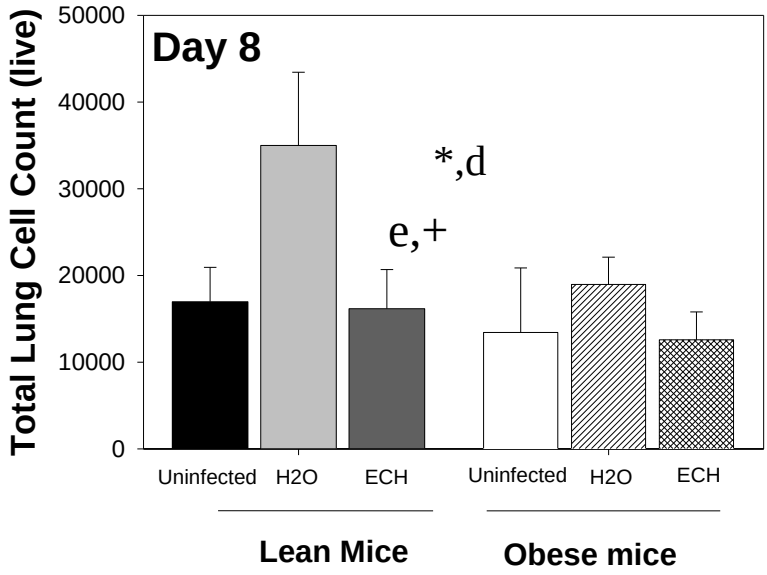
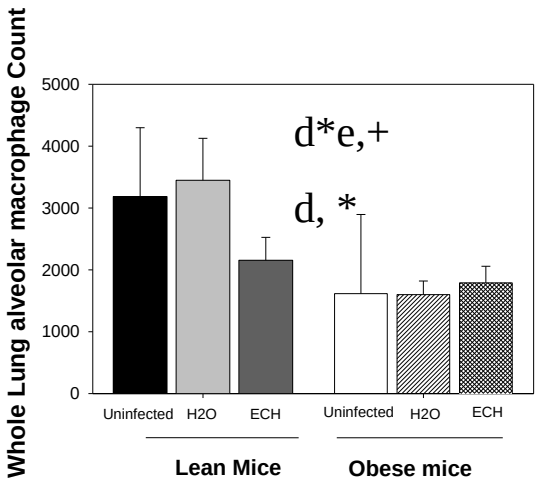


Figure 7. BAL cell populations from day 8 post infection. Mice were infected with A/PR/8/34 and treated with either ECH or H2O control. BAL was collected day 8 p.i. and analyzed for cell populations using flow cytometry. Percent of total cell counts are shown for each population +/-SEM. Effect of obesity was seen with decreases in alveolar macrophages % and CD8+ T cells % ($p<0.05$). Diet by Extract interaction was observed in the CD8% ($p=0.05$) with obese mice treated with ECH showing a decrease in CD8% whereas lean mice had an increase. A similar pattern was observed with respect to CD3+ cells, although this was only a trend ($p=0.09$). *, d $p<0.05$ obesity effect; +d $p<0.10$; obesity*extract interaction, d*e <0.05; +d*e, $p<0.10$. Uninfected n=3; H2O lean, n=4; ECH Lean n=5; H2O obese n=7; ECH obese n=3.

Figure 8.


Day 8
Total Lung Cell Count (live)
50000
40000
30000
20000
10000
0
*,d
e,+
Uninfected
H2O
ECH
Uninfected
H2O
ECH
Lean Mice
Obese mice


Whole Lung alveolar macrophage Count
5000
4000
3000
2000
1000
0
d*e,+
d, *
Uninfected
H2O
ECH
Uninfected
H2O
ECH
Lean Mice
Obese mice

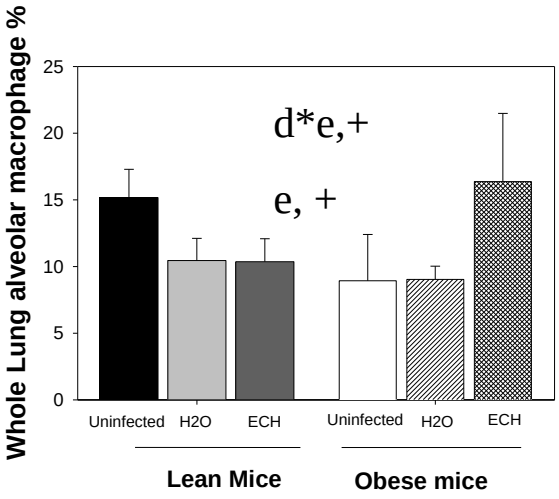

Whole Lung alveolar macrophage %
25
20
15
10
5
0
d*e,+
e, +
Uninfected
H2O
ECH
Uninfected
H2O
ECH
Lean Mice
Obese mice

Figure 8. continued.

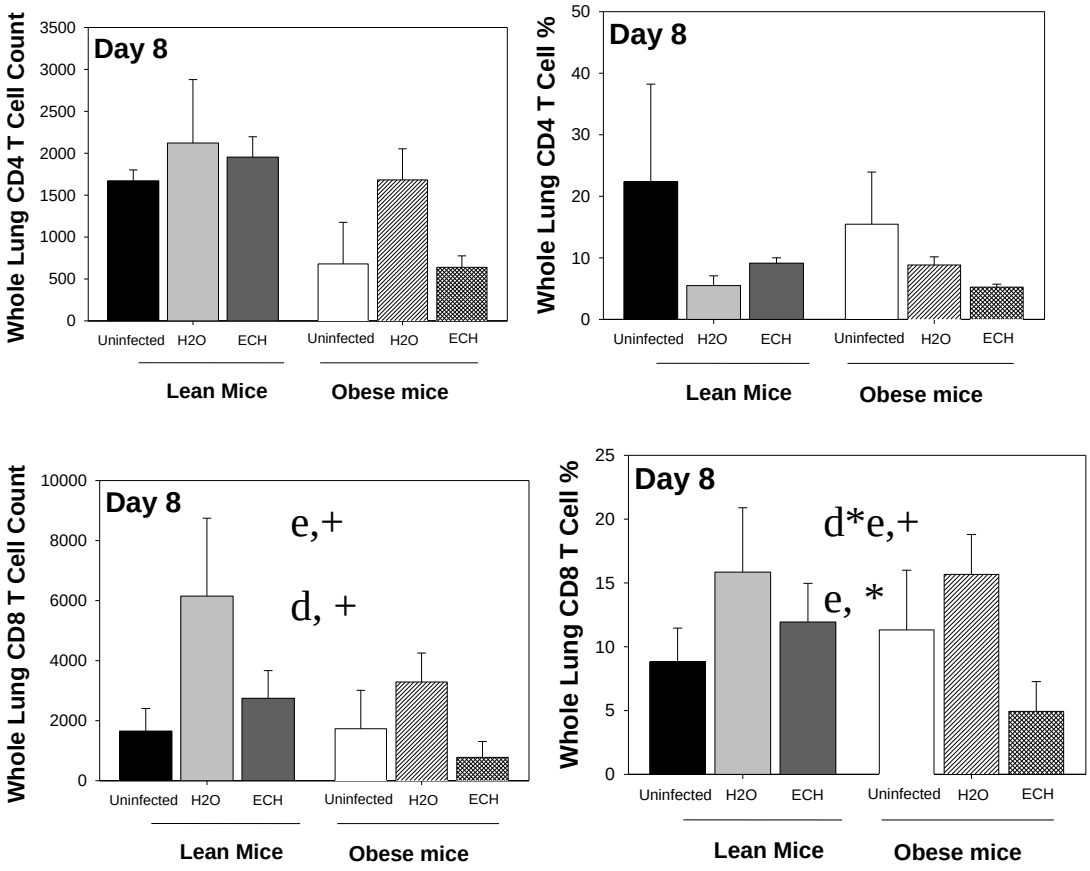


Figure 8. Whole lung cell populations. Mice were infected with A/PR/8/34 and treated with either ECH or H2O. Whole lungs were collected on day 8 p.i. Lung cells were isolated, cultured, and then identified using appropriate antibodies and flow cytometry. There was a trend towards a main effect of ECH with decreased total lung cell counts in mice receiving ECH treatment (e+) (p=0.07). In the whole lung, there was a diet by ECH interaction (d * e)for the alveolar macrophage count (p=0.09) and percent (p=0.10) such that lean mice treated with ECH tended to have a reduced number of macrophages whereas obese mice treated with ECH tended to have a greater percentage of alveolar

macrophages. There was a main effect of obesity with decreased alveolar macrophages count (d*, p<0.05). ECH treatment reduced CD8% in both lean and obese mice (e*, p <0.05) and tended to decrease total CD8 number (e+, p=0.07). Obese mice had decreased total lung cell count (d*, p<0.05), and trend towards fewer CD8% (d+, p=0.10) *, e p<0.05; +, e p<0.10; obesity effects d* p <0.05, d+ p=0.10; d, diet; e, extract; d*e, interaction p<0.05. Uninfected n=3, H2O lean, n=4; ECH Lean n=5; H2O obese n=7; ECH obese n=3.

Figure 9.

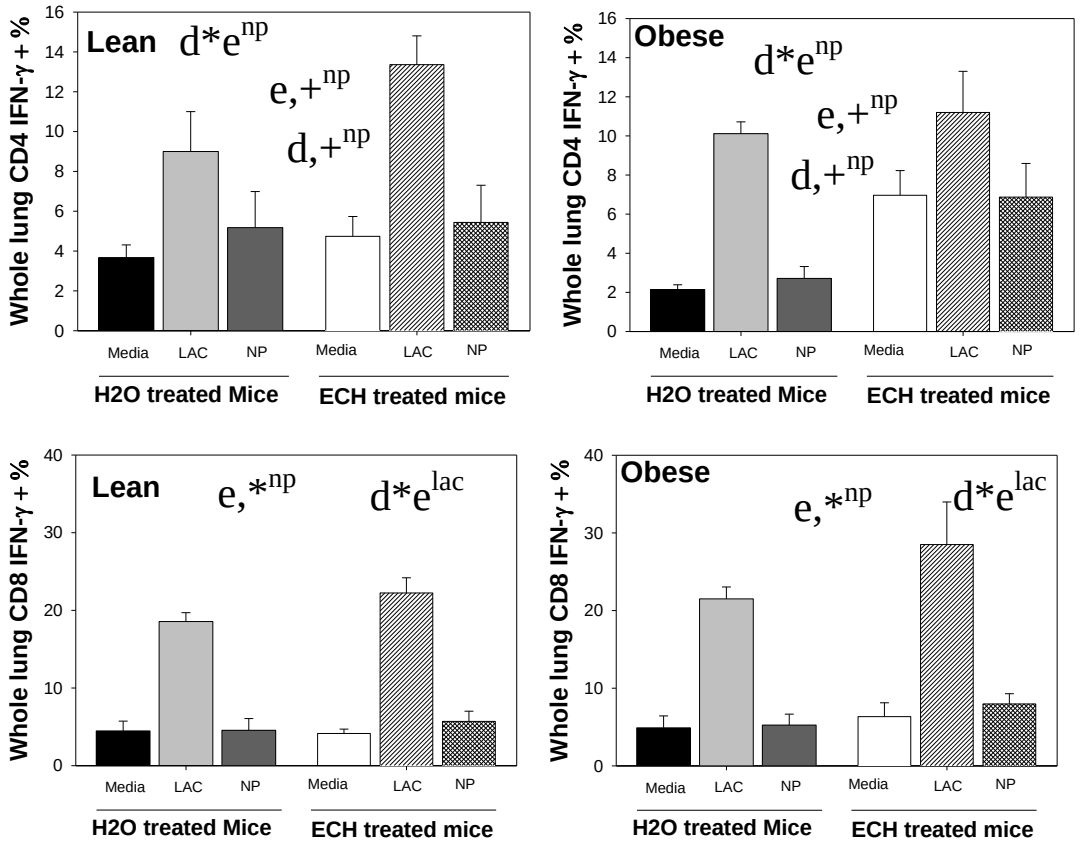


Figure 9. **Day 8 Intracellular flow cytometry NP, Med, and LAC stimulated lung cells.** Mice were infected with influenza A/PR/8/34 virus and treated with either ECH or vehicle control (H2O). Whole lungs were collected on day 8 p.i., cultured, and stimulated media only, leukocyte activation cocktail, or NP peptide from influenza virus. Cell populations and IFN-γ producing cells were analyzed using flow cytometry. Lean mice and obese mice %CD4 and %CD8 populations producing IFN-γ are shown. There was a diet*extract (d*e) interaction ($p<0.05$) with obese mice having increased CD4%IFN-γ+ cells when stimulated with media only. There was also a main effect of ECH in the media stimulation with ECH treated obese mice having increased CD8% cells producing IFN-γ ($p=0.08$). With the PMA-ionomycin (LAC) stimulation, the percent CD4+IFNγ+ ($p=0.07$) increased in obese mice treated with ECH (d*e $p=0.06$) and also had an increase production of CD8+IFNγ ($p<0.05$). There was a d*e interaction ($p<0.05$) with the NP stimulation. The main effect of obesity shows decreases in the CD4 T cells producing IFN-γ in obese mice ($p=0.05$) in response to NP stimulation. A main effect of extract ($p=0.07$) was also observed with ECH treated mice demonstrating an increase in CD4%IFNγ+. d*e ($p<0.05$ diet *ECH interaction) *, e ($p <0.05$ ECH effect); *e, (+ $p<0.10$ ECH effect). +, d (obesity effect $p<0.10$). np = NP peptide stimulated condition, lac = PMA-ionomycin stimulated condition. Uninfected n=3, H2O lean, n=4; ECH Lean n=5; H2O obese n=7; ECH obese n=3.

Figure 10.

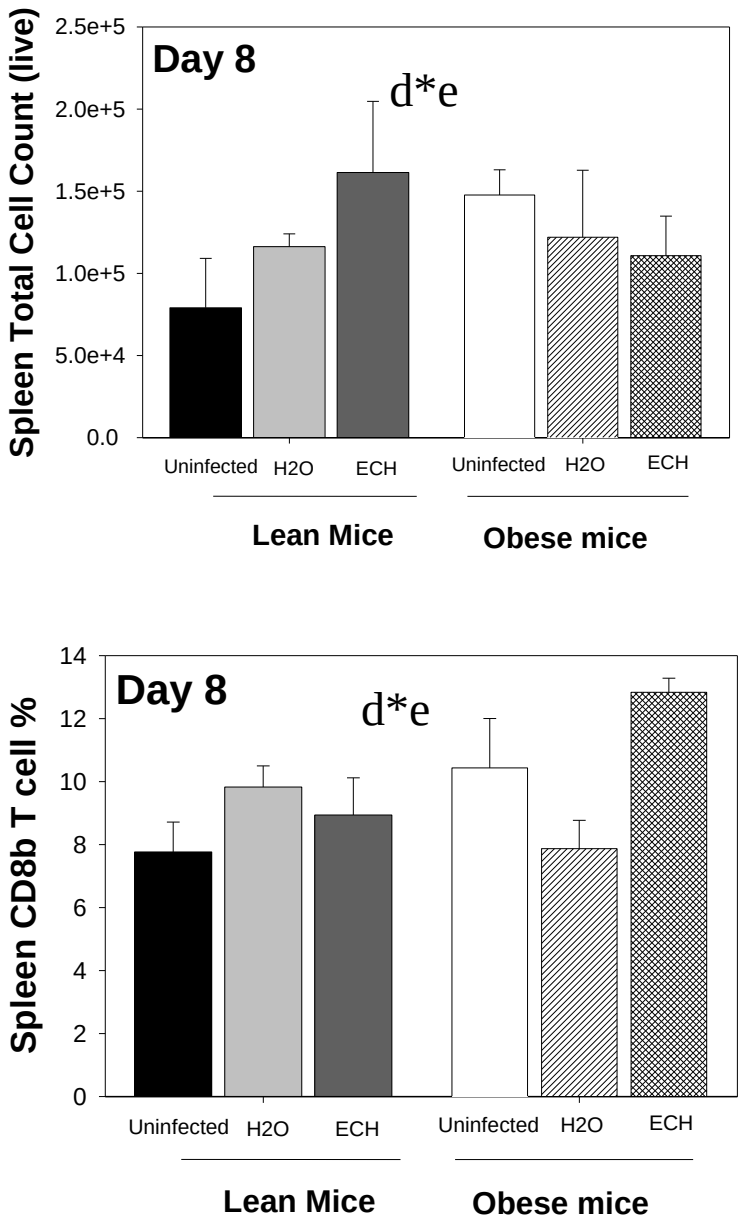


Figure 10. Day 8 p.i. Spleen cell populations. Mice were infected with A/PR/8/34 and treated with either ECH or H2O control. Spleens were collected day 8 p.i. and analyzed for cell populations using flow cytometry. Mean total spleen cell count +/- SEM, and CD8b as percent of total spleen cells. Total spleen counts were not different between the

2 groups. Significant interaction of obesity and ECH: %CD8+ in ECH obese mice was increased compared to lean mice that did not change with controls. d*e, $p<0.05$ Uninfected n=3, H2O lean, n=4; ECH Lean n=5; H2O obese n=7; ECH obese n=3.

Figure 11.

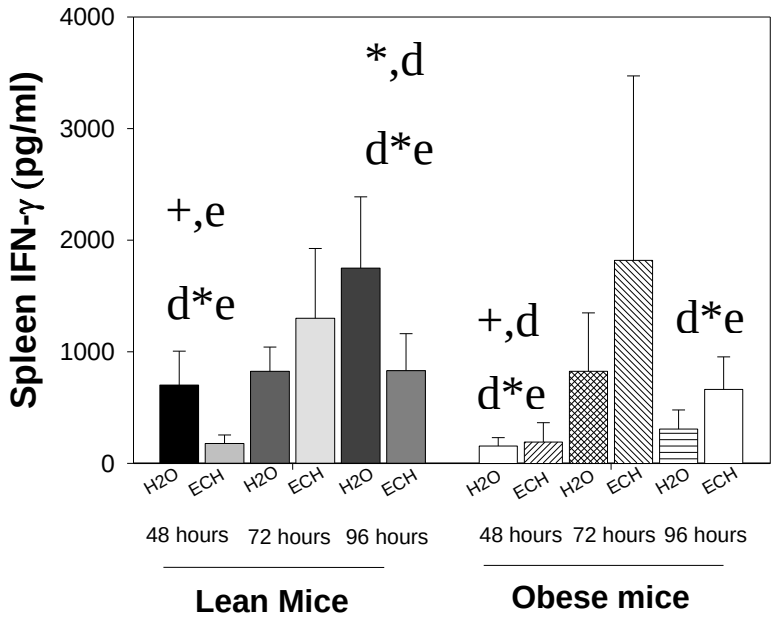


Figure 11. IFN-γ production by spleen cells stimulated with UV inactivated virus. Spleens were collected from mice infected with A/PR/8/34 on day 8 p.i. Spleen cells were isolated, cultured, and stimulated with UV inactivated A/PR/8/34. Supernatants were collected from the samples 48, 72, and 96 hours post-stimulation and analyzed for IFN-γ production by ELISA. A diet * ECH interaction was observed in the 48 ($p=0.07$) and 96 hour ($p=0.08$) IFN-γ production. At 48 hours, treatment with ECH tended to decrease IFN-γ production in lean mice ($p=0.08$), while no change was observed in the obese treatment group. ECH treatment increased IFN-γ production at 72 hours post but

resulted in a decline by 96 hours. A main effect of diet was observed at 48 hours and 96 hours. At both 48 and 96 hours obese mice had less IFN-γ production in the spleen cells ($p<0.05$); d*e, $p<0.05$, interaction of diet and ECH; *, d; main effect of diet $p<0.05$; +, e; main effect of ECH $p=0.08$. Uninfected n=3, H2O lean, n=4; ECH Lean n=5; H2O obese n=7; ECH obese n=3.

Table 1. Mouse diet nutritional information.

Diet Mouse group		**High fat** **DIO**		**Low fat** **Lean**	
protein	%	23.1		17.3	
fat	%	34.9		4.3	
fiber	%	6.5		4.7	
carbohydrates	%	25.9		67.4	
Minerals					
calcium	%	0.79		0.57	
phosphorus	%	0.59		0.43	
potassium	%	0.77		0.57	
magnesium	%	0.07		0.05	
sodium	%	0.15		0.12	
chlorine	%	0.25		0.21	
iron, ppm	ppm	64		44	
zinc, ppm	ppm	46		34	
manganese, ppm	ppm	76		55	
copper, ppm	ppm	7.8		5.7	
iodine, ppm	ppm	0.27		0.2	
Vitamins					
vitamin A	IU/g	5.2		3.8	
vitamin D	IU/g	1.3		0.9	
vitamin E	IU/g	67.2		49.3	
vitamin K	ppm	0.65		0.48	
thiamin	ppm	7.8		5.75	
riboflavin	ppm	8.7		28	
niacin	ppm	39		14	
folic acid	ppm	2.8		1.9	
biotin	ppm	0.3		0.2	
vitamin B12	mcg/kg	18		9	
ascorbic acid	ppm	0		0	
choline chloride	ppm	1290		950	
energy (kcal/g)		**kcal**	**%**	**kcal**	**%**
from:					
protein		0.924	18.1	0.692	18.3
fat		3.14	61.6	0.384	10.2
carbohydrates		1.035	20.3	2.697	71.5

DIO- diet induced obesity; ppm- parts per million; IU/g – international unit per gram; mcg/kg – microgram per kilogram; kcal/g – kilocalorie per gram.

Table 2. Flow cytometry cell populations

Day 3 BAL	**Flow marker**								
Cell	CD11b	CD11c	LyC	LyG	mPDCA	CD3	CD8a	SSC	AF
PMN	+	-	+	+				+	-
Mono	+	-	+	+				-	-
cDC		+	+	+					-
pDC	+	+	+	+	+				-
alveolar macrophage	+	+	-	-					+
CD8a T cell	-	-				+	+		-
CD3e+CD8a-	-	-				+	-		-
Day 8 BAL									
	CD11b	CD11c	Gr1	MHCII	CD3	CD8a	CD4	SSC	AF
PMN	+	-	+					+	-
Mono	+	-	+					-	-
DC	$+^{lo}$	$+^{int}$		$+^{int}$					-
xDC	$+^{lo}$	$+^{int}$			-	+			-
alveolar macrophage	+	+	-						+
CD8a T cell		-	-		+	+			-
CD4 T cell		-	-		+	-	+		-
Day 8 Spleen									
	CD11c	CD19	CD8b	CD4					
CD4 T cell	-	-	-	+					
CD8 T cell	-	-	+	-					
B cell	-	+	-	-					

BAL – Bronchoalveolar lavage; PMN- polymorphonuclear cell (neutrophil) cDC- conventional dendritic cell; pDC – plasmacytoid dendritic cell; xDC – cross presenting dendritic cell; int – intermediate; +, Antigen positive expression; -, no antigen expression. AF- autofluorescence.

Table 3. BAL cytokine and chemokine levels in Lean and Obese mice treated with H2O or ECH analyzed on day 3 and day 8 post-infection.

	Lean									
			Day 3				Day 8			
	Uninfected		H2O		ECH		H2O		ECH	
n=	3		3		4		4		5	
cytokines	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
IL-1a	31.35	9.53	52.17	30.07	48.81	23.61	*6.12	2.31	49.20	40.44
IL-1b	2.58	2.58	0.00	0.00	103.17	103.17	0.00	0.00	0.00	0.00
IL-2	14.98	2.51	8.12	5.75	*4.17	0.30	*2.84	0.92	11.05	7.83
IL-4	0.00	0.00	0.00	0.00	0.00	0.00	4.01	4.01	0.00	0.00
IL-5[D]	0.00	0.00	[D]0.17	0.17	[D]0.00	0.00	+70.71	36.15	*69.31	23.47
IL-6	0.00	0.00	*52.41	13.83	4636.59	3624.35	1661.27	966.53	+1724.18	641.08
IL-10	16.18	4.64	7.49	2.92	27.13	18.25	52.25	23.33	+62.39	17.30
IL-12p40	13.47	1.01	7.87	6.59	*0.15	0.15	*1.88	1.28	*3.05	2.31
IL-12p70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IL-15	6.13	2.39	2.41	1.77	+0.76	0.76	+2.29	0.76	+2.17	0.61
IL-17	0.00	0.00	0.00	0.00	3.22	3.22	5.66	5.66	+2.76	1.15
IFN-γ[d]	0.75	0.75	[d]2.39	1.81	[d]3.01	3.01	+4792.33	2243.45	+3937.46	1451.76
TNF-α[d]	0.00	0.00	[d]0.00	0.00	[d]12.41	12.41	15.03	7.77	+20.46	8.12
IP-10[D]	7.04	1.17	[D]*232.62	37.03	[D]+365.58	163.39	*1143.51	256.83	*1430.96	460.83
Eotaxin	4.87	2.75	5.77	3.22	55.12	33.60	90.41	50.70	+132.18	47.99
KC	16.72	1.85	*65.07	12.20	5569.80	4938.99	189.25	106.79	+113.78	33.98
MCP-1	0.00	0.00	+25.88	8.59	2218.75	2171.74	706.61	477.34	+592.32	256.75
MIP-1α	0.00	0.00	15.76	7.92	167.58	155.60	*54.16	16.02	*55.74	18.25
MIP-1β	0.00	0.00	*42.01	14.09	836.81	799.46	*104.62	35.10	+134.85	45.38
	Obese									
			Day 3				Day 8			
	Uninfected		H2O		ECH		H2O		ECH	
n=	3		3		3		7		3	
cytokines	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
IL-1a	33.67	11.88	26.56	4.65	30.84	15.01	+5.59	0.88	+5.88	3.32
IL-1b	0.00	0.00	2.58	2.58	8.27	8.27	1.11	1.11	0.00	0.00
IL-2	8.58	2.96	5.02	1.42	8.95	4.92	+0.82	0.38	3.01	2.35
IL-4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.32	12.32
IL-5[D]	0.00	0.00	[D]+4.17	2.09	[D]+3.46	1.84	*139.99	34.64	176.21	128.97
IL-6	1.37	1.13	469.52	375.07	5196.10	5095.36	*4460.34	1401.15	2628.83	1624.29
IL-10	10.81	2.99	7.35	2.08	13.40	9.16	+81.94	31.31	38.77	28.26

Table 3. continued.

cytokines	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
IL-12p40	10.14	3.64	8.64	4.86	8.61	6.00	+0.48	0.27	2.54	2.44
IL-12p70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IL-15	2.84	2.20	0.75	0.75	3.61	3.61	5.70	2.38	0.45	0.45
IL-17	0.00	0.00	0.00	0.00	0.00	0.00	1.53	0.98	0.68	0.68
IFN-γ^{d}	0.00	0.00	^{d}16.52	12.18	^{d}7.62	5.17	*5296.81	1961.75	6403.27	3907.95
TNF-α^{d}	0.00	0.00	^{d}1.91	1.91	^{d}32.95	28.47	*24.46	7.19	16.17	14.94
IP-10^{D}	5.46	0.62	^{D}+499.54	166.68	^{D}343.97	104.32	*1395.88	361.91	1326.83	824.72
Eotaxin	1.61	1.35	11.04	5.01	31.77	24.21	134.21	56.34	135.98	114.69
KC	15.65	3.89	225.23	171.15	11834.05	11750.12	*163.28	34.03	302.35	196.95
MCP-1	1.46	1.46	+300.53	265.25	*228.86	120.19	1024.47	485.47	980.60	890.15
MIP-1α	1.51	1.51	65.27	47.42	103.52	81.80	+66.10	22.66	48.76	34.01
MIP-1β	3.82	3.82	122.92	83.90	79.72	44.08	202.91	82.12	134.78	100.90

SEM – standard error of the mean. *, $p<0.05$ relative to uninfected, +, $p=0.1$ relative to uninfected. Effect of obesity, D, $p<0.05$; d, $p=0.1$ (day 3 only).

Table 4. Day 3 BAL cell count and cell subpopulations.

	DAY 3 BAL							
	Lean				**Obese**			
	H2O		**ECH**		**H2O**		**ECH**	
	MEAN	**SEM**	**MEAN**	**SEM**	**MEAN**	**SEM**	**MEAN**	**SEM**
Total Cells	16644.25	9310.05	19743.83	4942.19	16346.33	9449.64	21700.00	9951.05
iMono%	4.52	2.90	2.30	1.69	1.96	0.62	1.87	1.72
PMN%	0.08	0.07	0.04	0.01	0.19	0.14	0.13	0.12
cDC%^{e}	0.37	0.13	0.18	0.12	0.59	0.27	0.14	0.07
pDC%^{D}	3.53	0.55	3.23	1.23	1.36	0.32	2.00	0.90
CD3e%^{E}	4.47	1.07	1.32	0.22	3.97	1.02	1.03	0.40
CD8%	8.52	0.96	7.73	3.06	6.81	1.88	3.95	0.54

SEM – standard error of the mean. Effect of obesity, D, $p<0.05$ Effect of extract, E, $p<0.05$; e, $p=0.1$

Table 5. Day 8 BAL cell count and cell subpopulations.

	Day 8 BAL Cell Populations					
	Lean					
	Uninfected		H2O		ECH	
n=	3		4		5	
Cells	Mean	SEM	Mean	SEM	Mean	SEM
total cells	6229.67	1532.79	30380.25	14341.93	23080.60	4808.80
alveolar macrophages %[D]	33.13	4.30	36.08	2.04	31.80	3.19
xDC %	0.00	0.00	0.85	0.49	1.06	0.28
CD4%	0.23	0.09	3.80	0.66	4.96	1.21
CD3e%	5.77	2.26	1.58	0.43	3.70	1.33
iMono%	0.03	0.03	1.15	0.21	1.52	0.46
PMN %	0.00	0.00	0.58	0.11	0.78	0.25
CD8% [D*E, D]	0.40	0.06	2.90	1.31	4.66	0.46
	Obese					
	Uninfected		H2O		ECH	
n=	3		7		3	
Cells	Mean	SEM	Mean	SEM	Mean	SEM
total cells	4342.67	1361.30	36096.29	9586.01	21111.33	14328.08
alveolar macrophages %[D]	20.53	4.55	27.61	7.41	26.80	6.51
xDC %	0.03	0.03	0.69	0.29	0.17	0.09
CD4%	0.20	0.00	2.97	0.52	2.40	1.17
CD3e% [d*e]	3.47	0.52	1.50	0.32	2.73	1.87
iMono%	0.00	0.00	0.94	0.18	0.73	0.41
PMN %	0.00	0.00	0.44	0.14	0.37	0.27
CD8%	0.87	0.19	3.04	1.04	1.13	0.44

SEM – standard error of the mean. Diet and extract interaction D*E, $p<0.05$; d*e, $p=0.1$; effect of obesity D, $p<0.05$.

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CHAPTER V. SUMMARY AND CONCLUSIONS FOR THE DISSERTATION

Influenza is a seasonal respiratory infection that continues to cause significant morbidity and mortality in US and also worldwide. Although a vaccine is available for seasonal influenza, the match between viral antigens contained in the vaccine and the strain of virus circulating in the population is not always a perfect match. Also vaccine efficacy may be reduced in certain populations such as the elderly or obese. Anti-viral medications have been found to useful in treating the infection, but due to the high mutation rate and recombination of the Influenza A virus, several anti-viral medications have been discontinued due to high probability of influenza resistance. Given these limitations, along with the current interest in "alternative therapies" by the public, *Echinacea* has emerged as a popular remedy for treating colds and influenza. There nine known species of *Echinacea*, three of which are commonly used for medicinal purposes: *E. purpurea, E. angustifolia, and E. pallida.* A variety of preparations have been tested for immune enhancing properties. Most commonly, the root of the plant is used to make an extract that is either water-based or ethanol-based. Due to the chemical composition profile of each extract (water or ethanol), a variety of immune changes can be observed. In general, water-based extracts tend to exhibit immunomodulatory properties, whereas ethanol extracts are considered to have anti-inflammatory properties.

In this dissertation, we chose to examine the effect of different *Echinacea* extracts on infection with influenza virus. The overall goal of the research conducted as part of this dissertation was to extend the current knowledge base by developing an understanding of how *Echinacea* may impact innate or adaptive immune response to influenza, evaluate which types of extracts from which species may be most beneficial in

terms of enhanced immune response during influenza infection, and to establish whether *Echinacea* may have additional benefits in an obese model.

In Chapter II of this dissertation, we used an *in vitro* modeling system to evaluate the effect of *Echinacea* on influenza infection on viral load, lung epithelial cells, and spleen mixed immune cell populations from infected mice. In this study, we used three species of *Echinacea*: *E. purpurea*, *E. angustifolia*, and *E. paradoxa var. paradoxa*. First, we hypothesized that there would be a decrease in viral titer with the *Echinacea* treatments. To evaluate the effect of *Echinacea* on the viral titer, we utilized both water and ethanol preparations for each *Echinacea* species given at a high and low dose of each extract. We found that *E. purpurea* H2O extract demonstrated anti-viral activity *in vitro* at both low and high concentrations tested, whereas *Echinacea* EtOH extracts demonstrated anti-viral activity only at high concentrations of extract. Next, we evaluated the effect of 6 *Echinacea* extracts on the immune response in lung epithelial cells using A549 cell culture given at only one dose. Both the *Echinacea* water and ethanol extracts increased cytokines/chemokines production by A549 epithelial cells (eotaxin, GM-CSF, IL-8, MIP-1α/β), whereas the *Echinacea* ethanol extracts decreased production of IL-6 and IP-10. Finally to evaluate the effect of *Echinacea* treatment on immune cells, spleen cells from infected mice were cultured *in vitro*, treated with *Echinacea* extracts, and stimulated with virus. *E. purpurea* H2O extracts increased IL-2, IL-4, IL-5, and IL-17 as well as chemokines KC and MIP-1α/β. In contrast, the *Echinacea* EtOH extracts generally decreased cytokine response and demonstrated an anti-inflammatory profile. Based on these findings, it was concluded that *Echinacea* extracts may enhance the immune response to influenza infection by decreasing viral

load, enhancing the innate immune responses, and limiting influenza-associated inflammation.

The study presented in Chapter III was designed to evaluate the effect of using a combination of extracts delivered at specific times during the course of influenza infection in mice. In this chapter, we evaluated the effects of treating influenza infection with an immunostimulatory water extract at the early phase of infection, then treating with an anti-inflammatory ethanol extract at the late phase of infection. In this study, we used *E. angustifolia* water (*Eang* H2O) and ethanol (*Eang* EtOH) extracts and *E. paradoxa var. paradoxa* ethanol extract (*Epara* EtOH) and consisted of two separate experiments. In experiment 1, we evaluated *Eang* H2O/*Eang* EtOH treatment on immune response against influenza infection. In experiment 2, we evaluated the effect of *Eang* H2O/*Eang* EtOH and *Eang* H2O/*Epara* EtOH extract treatment on lung lesion scores and gene expression of immune parameters in the lung (utilizing the bioinformatics data mining program DAVID). In experiment one, we showed that *Eang* H2O */Eang* EtOH decreased cytokines IFN-γ and TNF-α in BAL of treated mice compared to the BAL of H2O/EtOH control mice. In addition, the concentration of multiple chemokines tended to be lower in the *Eang* H20/*Eang* EtOH mice including eotaxin, KC, MCP-1, RANTES, LIX, along with the cytokines IL-12p70 and IL-6, suggesting an overall reduction in inflammatory mediators by *Echinacea*. We also found higher serum antibody against influenza (IgG) in *Eang* H2O/*Eang* EtOH treated mice compared to vehicle control mice, suggesting enhancement of host immunity. In experiment two, five different treatment groups were used to compare the effects of two different species of *Echinacea*: H2O/EtOH, *Eang* H2O/EtOH, *Eang* H2O/*Eang* EtOH,

Eang H2O/*Eang* EtOH, and *Eang* H2O/*Epara* EtOH. No change in lesion scores or weight loss was observed in any of the treatment groups. DAVID bioinformatics analysis of the gene fold changes compared to H2O/EtOH controls revealed that the *Eang* H2O/EtOH treatment group up regulated more genes involved with lymphocyte activation and differentiation, whereas the *Eang* H2O/*Eang* EtOH and *Eang* H2O/*Epara* EtOH groups down-regulated expression of IL-8-like chemokines and genes involved in wound healing. While no changes were observed in illness severity measures, body weight and lesion score, there was a modulation of cytokine/chemokines in the BAL of treated mice compared to controls. Gene expression data analysis revealed different profiles for each combination analyzed suggesting that the combination selected could greatly impact the anti-inflammatory effects in the lung and potential benefit during influenza infections. Increased anti-influenza antibody at day 9 p.i. suggests a potential stimulation during the early phases of infection, and the cytokine/chemokine profile suggests an anti-inflammatory effect in the late phases of influenza infection for the mice treated with a combination of *Echinacea* water and *Echinacea* ethanol extracts. The DAVID analysis suggests that there is an immunomodulation in activation of immune cells that we were not able to detect with immune measures analyzed at day 8 or day 9, but may reflect changes that would be seen at later time points in the infection.

In Chapter IV, the effect of *Echinacea* treatment on influenza infection in obese mice was evaluated. Obesity is associated with immune dysfunction in both the innate and adaptive immune responses. Since *Echinacea* has been shown to modulate influenza infection by providing both immune enhancements and decreased lung inflammation, we hypothesized that treatment of diet induced obese mice with *Echinacea* during the course

of influenza infection would restore the impaired immune response seen with obesity. We chose to study *Echinacea purpurea* in obese mice because it one of the most commonly available extracts, and it also demonstrated increased anti-viral activity and enhanced immune activity in lung and spleen cells as shown by in the results we obtained in Chapter II of this dissertation. In this study, we found an overall effect of obesity at day 3 p.i. Infected obese mice had increased IL-5, IP-10 and IFN-γ in the BAL, but numbers of decreased plasmacytoid dendritic cells (pDCs), alveolar macrophages, and CD8+ cells. These findings are consistent with other studies in obese mice. The effects of *Echinacea* treatment at day 3 p.i. were decreased conventional dendritic cells (cDC) and CD3+ cells (number and percentage). At day 8 p.i., the effect of obesity (diet) was a reduction in alveolar macrophages, reduced CD8+ cells, and a decreased %CD4+IFNγ+ cells. Also at day 8 p.i., the *Echinacea* treated obese mice had decreased CD8+ counts and percentage and increased alveolar macrophage percentage in the lung at day 8 p.i. However, *Echinacea* treatment increased the production of IFN-γ in CD4 and CD8 cells when treated with PMA-ionomycin and with NP stimulation in both lean and obese mice. Although total spleen cell counts did not differ between the lean and obese mice, *Echinacea* tended to increase IFN-γ production in cultured spleen cells at 72 hours post stimulation, but not at 48 or 96 hours. IFN-γ production by spleen cells obtained from the obese mice was lower than the lean mice. There was an increase in the CD8+ cell population in the *Echinacea* treated obese mice spleens. This could result in the increased in IFN-γ production seen in the *Echinacea* treated obese mice and be a result of earlier influenza infection resolution. We concluded that the obesity effects seen in the diet induced obesity model used in this study correlated with the findings of previous

studies. To the best of our knowledge, no previous studies have evaluated the effect of *Echinacea* treatment in a diet induced obesity model of influenza infection. *Echinacea* treatment increased alveolar macrophage percentage, and decreased CD8 in the lungs of obese mice, and altered the IFN-γ production in spleen cells. Overall, *Echinacea* did not correct the obesity associated immune changes seen in diet induced obesity, such as impaired cellular recruitment (pDC and CD8) thought to lead to morbidity associated with obesity.

In conclusion, this dissertation further confirms the immunostimulatory properties of *Echinacea* water extracts and anti-inflammatory properties of *Echinacea* ethanol extracts *in vitro*. It also suggests that a combination of *Echinacea* extracts delivered at specific times during the course of influenza infection may provide enhanced immunity and reduced lung inflammation depending on the *Echinacea* species utilized. Lastly, *Echinacea* treatment during influenza infection in obese mice provided no immune benefit and may result in decreased CD8 cells necessary for viral clearance and infection resolution.

APPENDIX

DAVID output with Enrichment scores, p-values, Genes up- or down-regulated for each pathway in the enrichment cluster, and Bonferroni values. Gene regulation of extract combination treated mice compared to the gene regulation of vehicle control (H2O/EtOH) treated mice on Day 8 post infection.

Up-regulation *Eang* H2O/EtOH relative to H2O/EtOH			
Enrichment Score: 14.7			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0045321~leukocyte activation	0.00	PTPRC, ICOSL, CR2, CD8A, CD3E, PIK3CD, TLR4, VAV1, HDAC5, DOCK2, CXCR5, CCND3, CLCF1, GADD45G, CD4, IMPDH1, HELLS	0.00
GO:0001775~cell activation	0.00	PTPRC, ICOSL, CR2, CD8A, CD3E, PIK3CD, TLR4, VAV1, HDAC5, DOCK2, CXCR5, CCND3, CLCF1, GADD45G, CD4, IMPDH1, HELLS	0.00
GO:0046649~lymphocyte activation	0.00	PTPRC, ICOSL, CR2, CD8A, CD3E, PIK3CD, VAV1, HDAC5, DOCK2, CXCR5, CCND3, CLCF1, GADD45G, CD4, IMPDH1, HELLS	0.00
GO:0030098~lymphocyte differentiation	0.00	HDAC5, PTPRC, DOCK2, CD8A, CLCF1, CD3E, GADD45G, CD4, VAV1, HELLS	0.00
GO:0002520~immune system development	0.00	HDAC5, PTPRC, DOCK2, ICOSL, CXCR5, CD8A, CLCF1, CD3E, GADD45G, CD4, VAV1, HELLS	0.00
GO:0002521~leukocyte differentiation	0.00	HDAC5, PTPRC, DOCK2, CD8A, CLCF1, CD3E, GADD45G, CD4, VAV1, HELLS	0.00
GO:0042110~T cell activation	0.00	PTPRC, DOCK2, ICOSL, CCND3, CD8A, CD3E, GADD45G, CD4, VAV1	0.00
GO:0048534~hemopoietic or lymphoid organ development	0.00	HDAC5, PTPRC, DOCK2, CXCR5, CD8A, CLCF1, CD3E, GADD45G, CD4, VAV1, HELLS	0.00
GO:0030097~hemopoiesis	0.00	HDAC5, PTPRC, DOCK2, CD8A, CLCF1, CD3E, GADD45G, CD4, VAV1, HELLS	0.00
GO:0030217~T cell differentiation	0.00	PTPRC, DOCK2, CD8A, CD3E, GADD45G, CD4, VAV1	0.00
Enrichment Score: 5.1			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0030217~T cell differentiation	0.00	PTPRC, DOCK2, CD8A, CD3E, GADD45G, CD4, VAV1	0.00

GO:0009967~positive regulation of signal transduction	0.00	PTPRC, DOCK2, CD8A, CD3E, CD4, TLR4, IL11	0.00
GO:0010647~positive regulation of cell communication	0.00	PTPRC, DOCK2, CD8A, CD3E, CD4, TLR4, IL11	0.00
mmu05340:Primary immunodeficiency	0.00	PTPRC, CD8B1, CD8A, CD3E, CD4	0.00
GO:0045058~T cell selection	0.00	PTPRC, DOCK2, CD3E, CD4	0.00
GO:0045060~negative thymic T cell selection	0.00	PTPRC, DOCK2, CD3E	0.06
Enrichment Score: 4.9			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0046651~lymphocyte proliferation	0.00	PTPRC, DOCK2, CCND3, IMPDH1, HELLS	0.00
GO:0070661~leukocyte proliferation	0.00	PTPRC, DOCK2, CCND3, IMPDH1, HELLS	0.00
Enrichment Score: 3.8			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0030217~T cell differentiation	0.00	PTPRC, DOCK2, CD8A, CD3E, GADD45G, CD4, VAV1	0.00
GO:0006955~immune response	0.00	PTPRC, ICOSL, CR2, CD8B1, CD8A, IL27, GADD45G, CD4, TLR4, VAV1	0.00
immune response	0.00	ICOSL, CR2, CD8B1, CD8A, IL27, CD4, TLR4	0.00
GO:0009986~cell surface	0.00	PTPRC, ICOSL, CR2, CD8B1, CD93, CD8A, CD3E, CD4	0.00
mmu04640:Hematopoietic cell lineage	0.00	CR2, CD8B1, CD8A, CD3E, CD4, IL5RA, IL11	0.00
GO:0009967~positive regulation of signal transduction	0.00	PTPRC, DOCK2, CD8A, CD3E, CD4, TLR4, IL11	0.00
GO:0009897~external side of plasma membrane	0.00	PTPRC, ICOSL, CR2, CD8B1, CD8A, CD3E, CD4	0.00
GO:0010647~positive regulation of cell communication	0.00	PTPRC, DOCK2, CD8A, CD3E, CD4, TLR4, IL11	0.00
mmu04660:T cell receptor signaling pathway	0.00	PTPRC, CD8B1, CD8A, CD3E, PIK3CD, CD4, VAV1	0.00
GO:0050870~positive regulation of T cell activation	0.00	PTPRC, ICOSL, CD3E, CD4, SOCS5	0.00
GO:0050848~regulation of calcium-mediated signaling	0.00	CD8A, CD3E, CD4	0.00
domain:Ig-like V-type	0.00	ICOSL, CD8B1, CD8A, CD4	0.06
mmu04514:Cell adhesion molecules (CAMs)	0.00	PTPRC, ICOSL, CD8B1, CD8A, CD4	0.07
m_tcraPathway:Lck and Fyn tyrosine kinases in initiation of TCR Activation	0.00	PTPRC, CD3E, CD4	0.07
Enrichment Score: 3.4			
Term	**P-Value**	**Genes**	**Bonferroni**

GO:0051249~regulation of lymphocyte activation	0.00	PTPRC, ICOSL, CD3E, IL27, CD4, TLR4, SOCS5	0.00
GO:0002694~regulation of leukocyte activation	0.00	PTPRC, ICOSL, CD3E, IL27, CD4, TLR4, SOCS5	0.00
GO:0050865~regulation of cell activation	0.00	PTPRC, ICOSL, CD3E, IL27, CD4, TLR4, SOCS5	0.00
GO:0009967~positive regulation of signal transduction	0.00	PTPRC, DOCK2, CD8A, CD3E, CD4, TLR4, IL11	0.00
GO:0051251~positive regulation of lymphocyte activation	0.00	PTPRC, ICOSL, CD3E, CD4, TLR4, SOCS5	0.00
GO:0010647~positive regulation of cell communication	0.00	PTPRC, DOCK2, CD8A, CD3E, CD4, TLR4, IL11	0.00
GO:0002696~positive regulation of leukocyte activation	0.00	PTPRC, ICOSL, CD3E, CD4, TLR4, SOCS5	0.00
GO:0050867~positive regulation of cell activation	0.00	PTPRC, ICOSL, CD3E, CD4, TLR4, SOCS5	0.00
GO:0050863~regulation of T cell activation	0.00	PTPRC, ICOSL, CD3E, IL27, CD4, SOCS5	0.00
GO:0002684~positive regulation of immune system process	0.00	PTPRC, ICOSL, CR2, CD3E, CD4, TLR4, SOCS5	0.00
GO:0050870~positive regulation of T cell activation	0.00	PTPRC, ICOSL, CD3E, CD4, SOCS5	0.00
mmu05340:Primary immunodeficiency	0.00	PTPRC, CD8B1, CD8A, CD3E, CD4	0.00
GO:0045058~T cell selection	0.00	PTPRC, DOCK2, CD3E, CD4	0.00
GO:0032944~regulation of mononuclear cell proliferation	0.00	PTPRC, ICOSL, CD3E, IL27, TLR4	0.01
GO:0050670~regulation of lymphocyte proliferation	0.00	PTPRC, ICOSL, CD3E, IL27, TLR4	0.01
GO:0070663~regulation of leukocyte proliferation	0.00	PTPRC, ICOSL, CD3E, IL27, TLR4	0.01
GO:0032946~positive regulation of mononuclear cell proliferation	0.00	PTPRC, ICOSL, CD3E, TLR4	0.05
GO:0050671~positive regulation of lymphocyte proliferation	0.00	PTPRC, ICOSL, CD3E, TLR4	0.05
GO:0070665~positive regulation of leukocyte proliferation	0.00	PTPRC, ICOSL, CD3E, TLR4	0.06
GO:0050730~regulation of peptidyl-tyrosine phosphorylation	0.00	PTPRC, CD3E, CD4, IL11	0.06
GO:0042325~regulation of phosphorylation	0.00	PTPRC, CD3E, GADD45G, CD4, TLR4, IL11	0.07
GO:0019220~regulation of phosphate metabolic process	0.00	PTPRC, CD3E, GADD45G, CD4, TLR4, IL11	0.08

GO:0051174~regulation of phosphorus metabolic process	0.00	PTPRC, CD3E, GADD45G, CD4, TLR4, IL11	0.08
GO:0042129~regulation of T cell proliferation	0.00	PTPRC, ICOSL, CD3E, IL27	0.08
GO:0042108~positive regulation of cytokine biosynthetic process	0.00	ICOSL, CD3E, TLR4	0.22
Enrichment Score: 3.2			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0009986~cell surface	0.00	PTPRC, ICOSL, CR2, CD8B1, CD93, CD8A, CD3E, CD4	0.00
transmembrane protein	0.00	PTPRC, CR2, CXCR5, CD8A, PRLR, CD3E, CD4, IL5RA	0.00
GO:0009897~external side of plasma membrane	0.00	PTPRC, ICOSL, CR2, CD8B1, CD8A, CD3E, CD4	0.00
Signal	0.00	PTPRC, ICOSL, CR2, CD8B1, CD8A, CD3E, IL27, TLR4, INHA, IL11, PRLR, CD93, CLCF1, CD4, IL5RA	0.00
signal peptide	0.00	PTPRC, ICOSL, CR2, CD8B1, CD8A, CD3E, IL27, TLR4, INHA, IL11, PRLR, CD93, CLCF1, CD4, IL5RA	0.00
topological domain:Extracellular	0.00	PTPRC, ICOSL, CR2, CD8B1, CD93, CXCR5, CD8A, PRLR, CD3E, CD4, TLR4, IL5RA	0.02
disulfide bond	0.00	ICOSL, CR2, CD8B1, CD93, CXCR5, CD8A, PRLR, CD3E, CD4, TLR4, IL5RA, INHA	0.01
Glycoprotein	0.00	PTPRC, ICOSL, CR2, CD8B1, CD8A, IL27, TLR4, INHA, PRLR, CD93, CXCR5, CLCF1, CD4, IL5RA	0.02
disulfide bond	0.00	ICOSL, CR2, CD8B1, CD93, CXCR5, CD8A, PRLR, CD3E, CD4, TLR4, IL5RA, INHA	0.04
Enrichment Score: 3.1			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0009986~cell surface	0.00	PTPRC, ICOSL, CR2, CD8B1, CD93, CD8A, CD3E, CD4	0.00
GO:0009897~external side of plasma membrane	0.00	PTPRC, ICOSL, CR2, CD8B1, CD8A, CD3E, CD4	0.00
GO:0002252~immune effector process	0.00	PTPRC, ICOSL, CR2, CD8A	0.00
Enrichment Score: 2.3			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0007243~protein kinase cascade	0.00	PTPRC, PRLR, CLCF1, GADD45G, TLR4, SOCS5	0.03
GO:0042325~regulation of phosphorylation	0.00	PTPRC, CD3E, GADD45G, CD4, TLR4, IL11	0.07
GO:0019220~regulation of phosphate metabolic process	0.00	PTPRC, CD3E, GADD45G, CD4, TLR4, IL11	0.08

Enrichment Score: 2.1			
Term	**P-Value**	**Genes**	**Bonferroni**
innate immunity	0.00	CR2, IL27, TLR4	0.35
GO:0006954~inflammatory response	0.01	HDAC5, CR2, IL27, TLR4	0.98
GO:0006952~defense response	0.01	HDAC5, PTPRC, CR2, IL27, TLR4	0.98
GO:0045087~innate immune response	0.01	CR2, IL27, TLR4	1.00
GO:0009611~response to wounding	0.02	HDAC5, CR2, IL27, TLR4	1.00
Enrichment Score: 1.5			
Term	**P-Value**	**Genes**	**Bonferroni**
IPR008957:Fibronectin, type III-like fold	0.02	PTPRC, PRLR, IL5RA	0.89
Up-regulation *Eang* H2O/*Epara* EtOH relative to H2O/EtOH			
Enrichment Score: 4.2			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0046649~lymphocyte activation	0.00	HDAC5, PTPRC, DOCK2, CLCF1, PIK3CD, VAV1	0.00
GO:0045321~leukocyte activation	0.00	HDAC5, PTPRC, DOCK2, CLCF1, PIK3CD, VAV1	0.00
GO:0001775~cell activation	0.00	HDAC5, PTPRC, DOCK2, CLCF1, PIK3CD, VAV1	0.00
GO:0030098~lymphocyte differentiation	0.00	HDAC5, PTPRC, DOCK2, CLCF1, VAV1	0.00
GO:0002521~leukocyte differentiation	0.00	HDAC5, PTPRC, DOCK2, CLCF1, VAV1	0.00
GO:0030097~hemopoiesis	0.00	HDAC5, PTPRC, DOCK2, CLCF1, VAV1	0.00
GO:0042113~B cell activation	0.00	HDAC5, PTPRC, CLCF1, PIK3CD	0.00
GO:0048534~hemopoietic or lymphoid organ development	0.00	HDAC5, PTPRC, DOCK2, CLCF1, VAV1	0.00
GO:0002520~immune system development	0.00	HDAC5, PTPRC, DOCK2, CLCF1, VAV1	0.01
mmu04666:Fc gamma R-mediated phagocytosis	0.00	PTPRC, DOCK2, PIK3CD, VAV1	0.01
GO:0030183~B cell differentiation	0.00	HDAC5, PTPRC, CLCF1	0.11
Enrichment Score: 1.4			
Term	**P-Value**	**Genes**	**Bonferroni**
Cytokine	0.00	CLCF1, IL27, IL11	0.15
Down-regulation *Eang* H2O/EtOH relative to H2O/EtOH			
Enrichment Score: 6.8			
Term	**P-Value**	**Genes**	**Bonferroni**
Cytokine	0.00	IL1F8, TNF, CCL20, CCL9, CCL19, CCL4	0.00
GO:0005125~cytokine	0.00	IL1F8, TNF, CCL20, CCL9, CCL19, CCL4	0.00

activity			
Secreted	0.00	IL1F8, TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.00
GO:0006955~immune response	0.00	IL1F8, TNF, CCL20, CCL9, CCL19, CCL4	0.00
GO:0005615~extracellular space	0.00	IL1F8, TNF, CCL20, CCL9, CCL19, CCL4	0.00
GO:0044421~extracellular region part	0.00	IL1F8, TNF, CCL20, CCL9, CCL19, CCL4	0.00
GO:0005576~extracellular region	0.00	IL1F8, TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.00
Enrichment Score: 6.8			
Term	**P-Value**	**Genes**	**Bonferroni**
GO:0006954~inflammatory response	0.00	IL1F8, TNF, CCL20, CRP, CCL19, CCL4	0.00
GO:0009611~response to wounding	0.00	IL1F8, TNF, CCL20, CRP, CCL19, CCL4	0.00
GO:0006952~defense response	0.00	IL1F8, TNF, CCL20, CRP, CCL19, CCL4	0.00
Secreted	0.00	IL1F8, TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.00
GO:0005576~extracellular region	0.00	IL1F8, TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.00
Enrichment Score: 5.0			
Term	**P-Value**	**Genes**	**Bonferroni**
IPR000827:Small chemokine, C-C group, conserved site	0.00	CCL20, CCL9, CCL19, CCL4	0.00
PIRSF001950:small inducible chemokine, C/CC types	0.00	CCL20, CCL9, CCL19, CCL4	0.00
IPR001811:Small chemokine, interleukin-8-like	0.00	CCL20, CCL9, CCL19, CCL4	0.00
GO:0008009~chemokine activity	0.00	CCL20, CCL9, CCL19, CCL4	0.00
GO:0042379~chemokine receptor binding	0.00	CCL20, CCL9, CCL19, CCL4	0.00
Chemotaxis	0.00	CCL20, CCL9, CCL19, CCL4	0.00
SM00199:SCY	0.00	CCL20, CCL9, CCL19, CCL4	0.00
GO:0006935~chemotaxis	0.00	CCL20, CCL9, CCL19, CCL4	0.00
GO:0042330~taxis	0.00	CCL20, CCL9, CCL19, CCL4	0.00
GO:0007626~locomotory behavior	0.00	CCL20, CCL9, CCL19, CCL4	0.02
mmu04062:Chemokine signaling pathway	0.00	CCL20, CCL9, CCL19, CCL4	0.00
inflammatory response	0.00	CCL20, CCL19, CCL4	0.01
GO:0007610~behavior	0.00	CCL20, CCL9, CCL19, CCL4	0.09
Enrichment Score: 3.8			
Term	**P-Value**	**Genes**	**Bonferroni**
Secreted	0.00	IL1F8, TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.00
GO:0005576~extracellular region	0.00	IL1F8, TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.00
disulfide bond	0.00	TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.01
disulfide bond	0.00	TNF, CCL20, CRP, CCL9, CCL19, CCL4	0.01

Down-regulation *Eang* H2O/*Eang* EtOH relative to H2O/EtOH			
Enrichment Score: 6.3			
Term	**P-Value**	**Genes**	**Bonferroni**
cytokine	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
GO:0005125~cytokine activity	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
GO:0006955~immune response	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
GO:0005615~extracellular space	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
mmu04060:Cytokine-cytokine receptor interaction	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
GO:0006954~inflammatory response	0.00	CCL3, TNF, CCL8, CCL5, CCL4	0.00
GO:0044421~extracellular region part	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
GO:0009611~response to wounding	0.00	CCL3, TNF, CCL8, CCL5, CCL4	0.00
Secreted	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
GO:0006952~defense response	0.00	CCL3, TNF, CCL8, CCL5, CCL4	0.00
GO:0005576~extracellular region	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
disulfide bond	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
disulfide bond	0.00	CCL3, TNF, TNFSF14, CCL8, CCL5, CCL4	0.00
Enrichment Score: 5.3			
Term	**P-Value**	**Genes**	**Bonferroni**
IPR000827:Small chemokine, C-C group, conserved site	0.00	CCL3, CCL8, CCL5, CCL4	0.00
PIRSF001950:small inducible chemokine, C/CC types	0.00	CCL3, CCL8, CCL5, CCL4	0.00
IPR001811:Small chemokine, interleukin-8-like	0.00	CCL3, CCL8, CCL5, CCL4	0.00
GO:0008009~chemokine activity	0.00	CCL3, CCL8, CCL5, CCL4	0.00
GO:0042379~chemokine receptor binding	0.00	CCL3, CCL8, CCL5, CCL4	0.00
GO:0006954~inflammatory response	0.00	CCL3, TNF, CCL8, CCL5, CCL4	0.00
chemotaxis	0.00	CCL3, CCL8, CCL5, CCL4	0.00
SM00199:SCY	0.00	CCL3, CCL8, CCL5, CCL4	0.00
inflammatory response	0.00	CCL3, CCL8, CCL5, CCL4	0.00
GO:0009611~response to wounding	0.00	CCL3, TNF, CCL8, CCL5, CCL4	0.00
GO:0006935~chemotaxis	0.00	CCL3, CCL8, CCL5, CCL4	0.00
GO:0042330~taxis	0.00	CCL3, CCL8, CCL5, CCL4	0.00
GO:0006952~defense response	0.00	CCL3, TNF, CCL8, CCL5, CCL4	0.00
mmu04620:Toll-like receptor signaling pathway	0.00	CCL3, TNF, CCL5, CCL4	0.00

GO:0007626~locomotory behavior	0.00	CCL3, CCL8, CCL5, CCL4	0.01
GO:0007610~behavior	0.00	CCL3, CCL8, CCL5, CCL4	0.05
mmu04062:Chemokine signaling pathway	0.00	CCL3, CCL8, CCL5, CCL4	0.01

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