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I, Rama Dhenni B.S., hereby submit this original work as part of the requirements for the degree of Master of Science in Immunology.

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Role of Granzyme B in the Susceptibility to Secondary Bacterial Infection after Viral Infection

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Role of Granzyme B in the Susceptibility to Secondary Bacterial Infection after Viral Infection

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by
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ABSTRACT

Viral infections predispose the host to secondary unrelated viral or bacterial infections, which is best exemplified by secondary bacterial pneumonia post-influenza virus infection. In response to viral infection, host cytotoxic protease granzyme B is induced. Granzyme B expression is associated with decreased antigen presentation by dendritic cells (DCs), release of immunosuppressive TGF-β, extracellular matrix degradation, and increased inflammatory response. Hence, we hypothesized that granzyme B is involved in the susceptibility to secondary bacterial infection after viral infection. By using a mouse model of secondary *Streptococcus pneumoniae* infection following primary influenza virus or lymphocytic choriomeningitis virus (LCMV) infection, we aimed to assess the role of granzyme B in the susceptibility to *S. pneumoniae* after viral infection. In the present study, we showed that sublethal influenza virus infection in wild-type (WT) mice induces expression of granzyme B that was detectable extracellularly and intracellularly in the lungs. Influenza-infected granzyme B-deficient mice had similar morbidity and mortality as well as viral burden compared to infected WT mice. Importantly, when we infected WT and granzyme-B deficient mice with *S. pneumoniae* after 10 and 14 days of influenza virus infection, both group of mice showed similar morbidity and mortality. Furthermore, lung bacterial burden in mice were similar between WT and granzyme B-deficient mice when infected with *S. pneumoniae* 10 days after influenza virus infection. Moreover, we showed that WT and granzyme B-deficient mice had similar susceptibility to systemic secondary *S. pneumoniae* infection after 10 days of sublethal LCMV infection. In conclusion, our data show that viral-induced granzyme B is not required for enhanced susceptibility to secondary *S. pneumoniae* infection.
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CHAPTER I INTRODUCTION

Epidemiology of Concomitant Viral and Bacterial Infections

Viral infections have been shown to be one of the major factors that can predispose animals and humans to secondary unrelated viral or bacterial infections (Table 1) (1). One of the best examples of disease caused by concurrent or sequential infections is bacterial pneumonia in which preceding infection by respiratory virus such as influenza allow lethal invasion by otherwise non-fatal bacterial infections. Influenza and pneumonia are the leading cause of death in the United States and worldwide in the context of infectious disease. They ranked consistently among the top 10 causes of death in the United States, causing approximately 50,000 deaths each year (2, 3). The large mortality seen every year is not only because of the influenza virus infection but also because the viral infection can often lead to increased susceptibility to secondary bacterial infections (4).

Numerous epidemiological studies have shown that secondary bacterial infections were the main cause of severe influenza-associated disease and mortality. A retrospective analysis from specimens from the 1918 influenza pandemic has demonstrated that almost all fatal cases of pneumonia showed evidence of bacterial infection (5), while further epidemiologic studies during influenza pandemics and epidemics have showed that cases of bacterial pneumonias peaking concurrently with influenza activity (6–8). In various populations and settings worldwide over decades, there have been strong and consistent relationships between influenza and secondary bacterial infections, particularly due to Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus pyogenes, and Haemophilus influenzae (4). It has also been
established that secondary bacterial pneumonia complicating influenza infection has been noted to be more severe and prolonged, with higher mortality rates compared to influenza virus infection alone (9). The development of animal models for concomitant viral and bacterial infections has also been instrumental in studying the lethal synergism of these polymicrobial diseases, in which a number of mechanism likely contribute to the impairments in host defense and immune response of the respiratory tract against bacteria following viral infection (10). Collectively, these epidemiological and experimental reports suggest that viral infections and its associated immune response can affect the immune response required to counter bacterial infections. A better understanding of how viral infections alter the susceptibility to secondary bacterial infections is thus needed in order to develop novel approaches to the prevention and/or treatment of this polymicrobial disease.

**Table 1.** Viral infections can influence the outcome to secondary unrelated pathogens

<table>
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Adapted from (1). MCMV, murine cytomegalovirus; LCMV, lymphocytic choriomeningitis virus.
Influenza Virus

Biology and Pathogenesis

Influenza viruses are member of Orthomyxoviridae family which comprises six genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus, Isavirus, and recently described Quaranjavirus. This family of viruses are characterized by a negative-sense, single-stranded, and segmented RNA genome, with envelope derived from host cell membrane incorporating virus glycoproteins and non-glycosylated proteins (11). While influenza B and C viruses have limited host range, influenza A viruses infect a variety of animals, including humans, birds, swine, horses, and dogs. Influenza A viruses are further classified into subtypes based on the antigenicity of their hemagglutinin (HA) and neuraminidase (NA) molecules; currently, 17 HA subtypes (H1–H17) and 9 NA subtypes (N1–N9) are known while antigenic subtypes have not been identified for influenza B and C viruses (12). Most study on influenza virus have been focused on influenza A virus, because it is the most virulent among the three influenza virus types and is the most significant epidemiologically (13).

The influenza A virus RNA (vRNA) segments encode 11 proteins: hemagglutinin (H), neuraminidase (N), matrix protein 1 (M1), matrix/ion-channel protein 2 (M2), nucleocapsid protein (NP), non-structural protein 1 (NS1), non-structural protein 2/nuclear export protein (NS2/NEP), three polymerase proteins (PA, PB1 and PB2), and a pro-inflammatory/pro-apoptotic protein (PB1-F2) (12). The interaction of host cell sialic-acid bearing glycoconjugates with hemagglutinin begins the infection cycle by allowing binding to host cells. Upon binding at the cell surface, the virus is internalized by receptor-mediated endocytosis. The low pH in the endosome triggers fusion of the
viral and endosomal membranes, releasing the viral ribonucleoproteins (vRNPs, consisted of viral RNA, NP, PA, PB1, and PB2) into the cytoplasm. This vRNPs are transported into the nucleus where they serve as the template for transcription into viral mRNA and synthesis of new vRNA. New virus proteins are synthesized from viral mRNA, while the new vRNA is replicated through a positive-sense intermediate (complementary RNA [cRNA]). Newly synthesized complex of vRNPs are then exported from the nucleus to the assembly site at the apical plasma membrane, where virus particles bud and released (12).

Influenza virus replicates in the epithelial cells throughout the respiratory system from the upper and lower respiratory tract. Non-fatal influenza viral infections predominantly involve the upper respiratory tract and trachea, with fatal cases usually showing evidence of pneumonia. Within 20–40 hours following infection and replication in epithelial cells, influenza virus can cause multifocal destruction and desquamation that will leave basal epithelial cells, exposed basement membrane, and hyaline membrane formation (13, 14). Neutrophils are recruited in response to epithelial necrosis with inflammatory mononuclear cells and interstitial lymphocyte infiltrates follows at later stage. Airway epithelium starts to regenerate by day 5 which is marked by basal cell migration and differentiation into ciliated epithelium (13).

**Immune Response to Influenza Virus**

The first line of defense against influenza virus infection is provided by the airway epithelial cells by mechanical and biochemical barriers, which include ciliated epithelial movement, mucus production, antimicrobial enzymes and peptides, as well as
antioxidant proteins (15). Influenza virus that can overcome this defense can be rapidly recognized by infected epithelial cells through pattern recognition receptors (PRRs) that recognize viral RNA which is the major pathogen-associated marker pattern (PAMP) of influenza virus. These PRRs include Toll-like receptors (TLRs), cytosolic sensor retinoic acid inducible gene I (RIG-I), and the NOD-like receptor family pryin domain containing 3 (NLRP3) protein (16). Recognition of influenza virus by these PRR in infected epithelial cells and specialized patrolling cells such as plasmacytoid dendritic cells (pDCs) activates downstream expression of type I interferons, which leads to activation of interferon stimulated genes (ISGs). Activation of ISGs triggers antiviral state by inhibiting protein synthesis in infected cells which consequently limit virus replication and activating key components of the innate and adaptive immune systems, including antigen presentation and production of cytokines involved in activation of T cells, B cells, and natural killer (NK) cells (17).

Macrophages are the tissue resident mononuclear phagocyte cells responsible for ingesting dead host cells and pathogens. In an experimental mice model, depletion of lung-resident macrophages (alveolar macrophages, AMs) prior to, but not 3 or 5 days following, influenza virus challenge results in uncontrolled viral replication and a significant increase in mortality suggesting that AMs play an important role in the early control of influenza virus infection before the adaptive immune responses is triggered (18). Phagocytosis has been suggested to be the key mechanism for AMs to limit influenza virus infection as demonstrated by Hashimoto et al., in which changes in the level of phagocytosis and the amount of virus in lung tissue of influenza-infected mice
correlated with each other (19). Thus, the results above suggest an indispensable innate immune response role of alveolar macrophages to influenza virus infection.

Other important effector cells of the innate immune response during influenza virus infection are NK cells which are able to directly kill virus-infected cells and participate in influencing the adaptive immunity through secretion of IFN-γ. Influenza virus that are bound to antibodies can be recognized and killed by NK cells through their Fc receptor, a process called antibody-dependent cell cytotoxicity (ADCC) (20). NK cells can also recognize influenza virus HA on infected cells with their natural cytotoxicity receptors NKp46, in which upon binding to the influenza virus HAs the receptors trigger the NK cells to lyse the infected cells (21).

Dendritic cells (DCs) are considered the professional antigen-presenting cells which play a critical role in connecting innate and adaptive immune responses upon influenza virus infection. DCs are distributed throughout the respiratory tract, including the airway epithelium, lung parenchyma, and the alveolar spaces of the lung, where they continuously survey for invading pathogens or foreign material (22). DCs can acquire antigen through two distinct mechanisms: by direct infection with IAV or through phagocytosis of either dead or dying epithelial cells. Upon activation of DCs, they migrate to the draining lymph node where they activate T cells by presenting the influenza virus antigen (23). Lung-resident DCs are a heterogeneous population that can be distinguished by their surface marker phenotype and function. Airway and alveolar DC (aDCs) and interstitial DC (iDCs) are the predominant DCs in the naïve lung which can be distinguish by their surface marker. Following influenza virus infection, there is a significant influx of aDCs and iDCs numbers, as well as the
recruitment of additional subsets such as inflammatory monocyte-derived DC, plasmacytoid DC (pDC), and CD8α+DC (22).

The adaptive immune response acts as the second line of defense and has been shown to be required for the control of influenza virus infection. The adaptive immune system is primarily activated by the antigen-presenting DCs that migrate from the respiratory tract to the lymph nodes or spleen. Two important components that induced by the antigen-presenting cells are virus-specific neutralizing antibodies produced by B cells and cytotoxic CD8 T cells. Antibodies against two surface glycoproteins of influenza virus, HA and NA, are of importance as the presence of antibodies specific for these glycoproteins correlates with protective immunity against influenza virus (24). The HA-specific antibodies bind to HA glycoproteins which results in inhibition of virus attachment and entry into host cells. Furthermore, HA- and NA-specific antibodies can also facilitate antibody-mediated phagocytosis or ADCC of the virus by phagocytic (neutrophils) or cytotoxic cells (NK cells) (24). Production of antibodies against influenza virus by B cells is driven by cytokines from a subset of CD4 T cells, T helper 2 (Th2) cells (23). In the absence of CD8 T cells, CD4 T cells, or B cells in mouse models of primary infection, less pathogenic isolates of influenza virus can be cleared. However, both CD4 and CD8 T cells, or B and T cells together are required for complete clearance of infection with more pathogenic isolates of influenza (24, 25). Hence, complete resolution of highly pathogenic influenza viral infection may require complex interactions of cellular and humoral immune responses.

The main function of virus-specific CD8 T cells is recognition of infected cells via viral peptide presented in the context of major histocompatibility complex class I (MHC-
I) and subsequent killing facilitated by perforin or Fas-dependent lysis. CD8 T cells are activated by DCs in the lymphoid tissues and recruited to the site of infection upon influenza virus infection in the lung, where they can further interact with recently recruited viral antigen-displaying inflammatory DCs (26). This second round of interaction with DCs in the infected lung tissue is important for an efficient antiviral CD8 T cell response because in vivo depletion of lung DCs during influenza virus infection led to increased mortality and sustained higher viral titers which correlates with impaired pulmonary CTL response (27). Upon recognizing virus-infected cells, CD8 T cells eliminate infected cells to prevent production of virus which is mediated by the release of perforin and the cytotoxic protease, granzymes. Perforin acts as a pore forming protein which permeabilizes the membrane of the infected cells to facilitate granzyme delivery into the cell to induce apoptosis. CD8 T cells also induce apoptosis of infected cells through perforin-independent Fas/FasL pathway, as perforin-deficient CD8 T cells are still able to induce cell lysis (28). Furthermore, activated CD8 T cells produce cytokines that display antiviral activity and improve antigen-presentation by stimulating MHC expression (23).

**Lymphocytic Choriomeningitis Virus (LCMV)**

**Biology and Pathogenesis**

Lymphocytic choriomeningitis virus (LCMV) is the first member of Arenaviridae family to be isolated and described. It was first discovered in 1933 from a St. Louis encephalitis epidemic (29) and was found to be a cause of aseptic meningitis (30). LCMV is a natural mouse pathogen that commonly used as a model system to study
immune responses and mechanisms of viral pathogenesis, rather than studied as a human pathogen. Most human infections are subclinical, but can display symptom such as fever, myalgia and headaches, and sometimes as advanced aseptic meningitis (31).

LCMV is an enveloped virus studded with evenly spaced glycoprotein projections that consist of viral glycoproteins GP1 and GP2. The viral nucleocapsid contains a bisegmented RNA genome consisting of two negative single-stranded RNA segments: a small (S) RNA encodes the viral glycoprotein precursor (GPC) and the nucleoprotein (NP); and a large (L) RNA which encodes the viral RNA polymerase and a small RING finger protein Z. The GPC is further cleaved into GP1 and GP2, in which GP1 is responsible for binding the virus to the cellular receptor α-dystroglycan (32). The main LCMV cellular receptor α-dystroglycan (α-DG) is highly conserved and widely expressed in adult host tissue, including skeletal muscle, salivary gland, pancreas, small intestine, liver, trachea, and kidney as well as specific cell type such as DCs (33). This receptor facilitates entry of LCMV virion by endocytosis and release by a pH-dependent membrane fusion into cytoplasm where LCMV replicates.

LCMV is a relatively non-cytopathic virus. Intraperitoneal LCMV infection of weanling mice results in interstitial inflammatory infiltration of liver and lung with high titer of virus in the spleen but not in the kidney (34). In infections by any route, the macrophage is usually identified as an early and prominent cell infected with many epithelial cells are later infected as infection spreads. The marginal zone and lymphoid follicles of the spleen and lymph nodes are commonly infected, in which antigen-presenting cells destruction by CTL in this organs is associated with the impairment of the effective immune response generation. Although there is an extensive involvement
of different cell types infected throughout the body, the pathological changes are relatively subtle, with comparatively little necrosis (32).

**Immune Response to LCMV**

The immune response to control LCMV infection is predominantly facilitated by T and B cells, although the presence of fully functional, differentiated CD8 T cells is indispensable to efficiently control the infection. In addition to viral clearance, the CD8 T-cell response is also responsible for induction of immunopathologies which appear to be mediated via granule secretory (perforin and granzymes) and the FasL/Fas interaction pathways (35). Nevertheless, the outcome of infection of the mouse with a given LCMV strain depends on the age and immunocompetence of the host, the route of inoculation, and the genetic background of the mouse. Infection of adult mice with LCMV Armstrong strain results in an acute infection that is cleared within 1 to 2 weeks in a perforin-mediated CD8 T cell-dependent manner, while infection with the clone 13 variant of Armstrong strain results in persistent viral infection that cannot be cleared by the CD8 T cells which is associated with higher affinity of the clone 13 strain to α-DG-expressing DCs (36). Similar to LCMV Armstrong strain, infection with hepatotopic LCMV WE strain results in acute infection in adult immunocompetent mice (37).

B cells and/or LCMV-specific neutralizing antibodies play a limited role to clear acute primary infection, but may play a substantial role in decreasing viral load and act synergistically with antiviral T cells to prevent reinfection and eradication of persistent LCMV infection. B cell-deficient mice are able to clear a low-dose LCMV challenge, but are unable in combating virus following high-dose challenge. Recovery from acute
LCMV infection consistently leads to long-term T cell and B cell memory and persistence of antiviral serum antibody for the life span of immunocompetent mice (36).

Although NK cells are activated to a high level of cytotoxicity, proliferate, and increased in number during LCMV infection, NK cell depletion had no effect on virus titers in the early stages of acute LCMV infection or during persistent LCMV infection (38). Mice depleted of NK cells had similar amounts of LCMV in their spleens, suggesting minimal contribution of NK cells to viral control in immunocompetent mice (38, 39). Recent studies however demonstrated that NK cells can regulate the antiviral CD8 T cells which control LCMV pathogenesis and persistence via perforin-mediated elimination of activated CD4 T cells (40).

**Streptococcus pneumoniae**

**Biology and Pathogenesis**

*Streptococcus pneumoniae* is an encapsulated, facultative anaerobe Gram-positive bacteria. Based on the hemolytic properties, *S. pneumoniae* is categorized as α-hemolytic because of its ability to produce hydrogen peroxide which converts hemoglobin into methemoglobin. Strains of *S. pneumoniae* can be classified into 90 different serotypes based on the fine chemical structure of the polysaccharides capsule. This polysaccharide capsule surrounds a cell wall that consists of peptidoglycan and lipoteichoic acid (LTA). Serotypes of *S. pneumoniae* vary in its invasiveness and ability to colonize, with serotypes 3, 6A, 6B, 9N, 19F, 23F, 31 being the major serotypes found in fatal cases of infection (41, 42). *S. pneumoniae* produces a range of virulence factors and cell wall-associated proteins, including the toxin pneumolysin that has pleiotropic
effects on the host immune response including cell lysis, complement activation, inhibition of neutrophil function, and increased inflammation, as well as opsonophagocytosis inhibition (43).

Pneumonia is the most common manifestation of *S. pneumoniae* infection, with *S. pneumoniae* being identified in 30–50% of pneumonia cases (44). The pathogenesis of pneumococcal pneumonia can be generally described into three clinical stages. First, as the bacteria invade the alveolus, the host cells respond to bacterial peptidoglycan while capillaries, and epithelial cells become leaky, and edema fluid spreads through the interalveolar connections. The second stage is characterized with the appearance of erythrocytes in the alveolus, clotted exudate throughout the lobe, and a dense lung with little internal air. In the final stage, patients appear toxic with poor pulmonary perfusion as leukocytes migrate into the lesion. This stage represents the tipping point at which the host response either overcomes bacterial multiplication or succumb to infection (45). The case fatality of pneumococcal pneumonia is 5–10% and increases to 20% in the presence of bacteremia. In addition to the bloodstream invasion, infection can rarely extend to involve the pericardial and pleural cavities, resulting in empyema (46).

**Immune Response to *Streptococcus pneumoniae*  

Bacterial invasion in the lung is first recognized by TLRs and other host receptors through bacterial pathogen-associated molecular patterns (PAMP) which serves to recruit and activate innate immune cells to the site of infection. In response to TLR signaling, alveolar epithelial cells produce inflammatory cytokines. As a result of direct
and indirect PAMP signaling, macrophages, neutrophils, and specific T-cell subsets are recruited to the lung and airways which promote inflammation and bacterial uptake (44).

Alveolar macrophages (AMs) and dendritic cells are the first line of defense in phagocytosis of bacteria and are important in coordinating the innate response to infection (47, 48). AMs are the major source of TNFα which, together with IL-1 (α and β), is important to induce the nuclear translocation of NF-κB transcription factors which eventually signal for neutrophil recruitment (48, 49). In established pneumonia, AMs are no longer important for bacterial clearance as neutrophils become the major cell phagocytosing bacteria. Rather, AMs contribute to regulate the inflammatory response via elimination of apoptotic neutrophils and AMs (50, 51).

Neutrophils are extremely important to achieve S. pneumoniae clearance and crucial for resolution of pneumococcal pneumonia, which is facilitated by the ability of neutrophils to adhere to blood vessel walls, chemotaxis, phagocytosis, and microbial killing (52, 53). Neutrophils especially recognize complement and immunoglobulins-opsonized S. pneumoniae, in which it can bind and ingest the microorganisms through phagocytosis. Phagocytized bacteria are killed by the action of NADPH oxidase found in the phagosome membranes which generates superoxide. In addition, neutrophils release multiple anti-microbial molecules from their granules, which include: myeloperoxidase, cathepsin G, neutrophil elastase, protease 3, and azurocidin (53).

The adaptive immune system provides a highly specific immune response that is initiated after exposure to the S. pneumoniae. Polysaccharide from S. pneumoniae induce a weak adaptive immune response as these antigens are unable to recruit cognate CD4+ T cell help through recognition of peptide–MHC class II complexes on
the surface of antigen-presenting cells to assist B cell antibody responses (54).
Therefore, immunity to S. pneumoniae after nasopharyngeal colonization in mice is dependent on antibody that recognizes several S. pneumoniae cell wall proteins (55). These antibodies facilitate AMs and neutrophil phagocytosis directly through Fc gamma receptors or by increasing complement activity against S. pneumoniae. Th17, a subset of CD4 T cells, are important for rapid recruitment of neutrophils to the lungs during S. pneumoniae invasion, while T-regulatory CD4 cells help regulate inflammatory responses and thereby may help preserve epithelial barrier integrity and prevent bacteremia (43, 56). CD8 T cells have been shown to be important for resistance against serotype 3 of S. pneumoniae, but not for serotype 2 and 8, possibly by reducing CD4 T cell-mediated inflammation (57).

During the resolution phase of S. pneumoniae lung infection, a dramatic increase of γδ T-cell population has been observed (58, 59). Infected mice deficient in γδ T-cells (TCRδ−/−) have increased AMs and DCs in the lungs, even after pneumococci have been cleared, suggesting that their elevated numbers in the lung most likely serve to limit the recruitment of professional antigen-presenting cells during the latter stages of infection (59).

**Mechanisms of Influenza Virus-Induced Susceptibility to Secondary Bacterial Pneumonia Infections**

Several mechanisms have been proposed to explain the increase in susceptibility to bacterial pneumonia succeeding influenza virus infection of the respiratory tract (60). Traditionally, the common accepted mechanism that responsible for increased
susceptibility to secondary bacterial infection is that influenza virus induces damage to the epithelial barrier in respiratory tract which provides increased available attachment sites for bacteria (61–63). Influenza virus infection induces activation of the innate and adaptive immune response which consequently stimulates a network of cytokines and chemokines that may impair the recruitment and activity of effector cells required to prevent bacterial invasion. Many studies have also demonstrated that dysregulated cytokine responses during influenza virus infection could impair host defense against subsequent bacterial invasion.

**Altered Bacterial Adherence to Respiratory Epithelium After Viral Infection**

Non-lethal influenza virus infection in mice results in distinct phenotypic changes in respiratory epithelial cells occurring as early as 24 hours' post-infection, with regeneration take place within 5 days (64). The cytotoxic nature of influenza virus causes injuries in the trachea and lung that allow pneumococcal bacteria to invade lung tissue as evidenced in early *in vivo* studies (61, 65). *In vitro* observations have also confirmed the increased ability of *H. influenzae* and *S. pneumoniae* to adhere to both primary respiratory epithelial cells and immortalized cell lines after influenza virus infection (66). Influenza virus neuraminidase activity which cleaves terminal sialic-acid moieties in the surface of respiratory epithelium has been shown to increase pneumococcal adhesion and colonization by exposing preferred bacterial ligands and underlying host cell surface (67). In addition, cell surface molecules such as platelet-activating factor receptor (PAFR), a receptor utilized by *S. pneumoniae* to invade respiratory epithelium, has been shown to be upregulated during influenza virus
infection which lead to increased pneumococcal adherence. PAFR-deficient mice have significantly reduced bacterial outgrowth in their lungs, diminished dissemination of the infection, and prolonged survival after secondary bacterial infection following influenza virus infection (68).

**Impaired Neutrophil and Macrophages Recruitment and/or Activation**

Several studies have been done to examine how influenza virus infection affects the number and recruitment of macrophages and neutrophils in the respiratory tract to fight subsequent bacterial infection. Influenza virus infection caused the decrease of more than of 90% resident alveolar macrophages after one week of influenza virus infection in mouse model, which correlates to the impaired clearance of secondary bacterial infection (69). Earlier studies have shown that influenza virus infection suppresses macrophage recruitment and bacterial killing activity (70, 71). Other studies have demonstrated that the phagocytic capacity of alveolar macrophages is enhanced early during viral infection but decreased significantly at later time points which leads to susceptibility to secondary bacterial infection (19, 72). The cytokine-mediated downregulation of the scavenger receptor MARCO is associated with temporally impaired AM phagocytic function (72).

Many studies have also demonstrated a reduced early neutrophil recruitment after bacterial challenge in animals previously infected with influenza virus, although later neutrophil accumulation (24h post bacteria) is not affected or even increased (73–75). Nevertheless, there are defects in the function of theses recruited neutrophils, including decreased phagocytic activity, myeloperoxidase production, respiratory burst,
and secretion of lysozyme (75–77). Furthermore, neutrophil apoptosis is increased when treated \textit{in vitro} with both influenza virus and \textit{S. pneumoniae} (78). However, this effect may not be significantly important as many of the clinical cases of secondary bacterial infection occurs after influenza virus infection and/or disease has been resolved. Additionally, depletion of neutrophils resulted in increased susceptibility to \textit{S. pneumoniae} in mice infected with influenza for 3 days. However, neutrophil depletion did not affect resistance to \textit{S. pneumoniae} in mice infected with influenza for 6 days, suggesting that neutrophil function was compromised by the influenza infection to a point similar to that caused by neutrophil depletion. Thus, collectively these data indicate that both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary \textit{S. pneumoniae} infection (77).

**Dysregulated Cytokine Responses**

Dysregulated cytokine and chemokine production during primary influenza infection can both promote deleterious tissue injury and impair innate responses required for effective clearance of subsequent bacterial invasion. Enhanced production of cytokines, including IFN-\(\alpha\), IFN-\(\beta\), IFN-\(\gamma\), IL-1\(\alpha\), IL-1\(\beta\), TNF-\(\alpha\), IL-6, IL-8, IL-10, TGF-\(\beta\), and chemokines including CCL2 (MCP-1), CCL3 (MIP-1\(\alpha\)), and CCL4 (MIP-1\(\beta\)), have been observed in lungs of animals and humans infected with influenza (79).

IFN pathway has been shown as an important mechanism by which influenza virus suppresses host defense against secondary bacterial infections. It is well known that type I IFNs (\(\alpha\) and \(\beta\)) and type II IFN (\(\gamma\)) are essential part of the innate cytokine
response to numerous viral infections, although in the context of influenza virus infection, there is a minimal contribution for IFN-α/β or IFN-γ pathways in protection or recovery from influenza virus infection in mice (80). No major effect on overall lethality, virus replication, kinetics of the cellular immune, was detectable in IFN-α/β receptor (IFNAR) and IFN-γ receptor knockout mice compared to its wild-type counterpart when infected with influenza virus. Interestingly, IFNAR knockout mice were protected from secondary bacterial infection after primary influenza virus infection, in which elevated CXCL1- and CXCL2-induced neutrophil recruitment during co-infection was thought to be a potential mechanism for increased sensitivity to secondary bacterial pneumonia (73). Type I IFN induced by influenza virus has also been shown to inhibit the generation of Th17 immunity which is critical for neutrophil recruitment and clearance of Staphylococcus aureus and S. pneumoniae from respiratory tract (81, 82). The inhibition of Th17 activation was shown to be dependent on inhibition of IL-23 production by DCs (81). Furthermore, adoptive transfer of IFNAR knockout γδ T cells into lungs of wild-type mice reduced their susceptibility to secondary S. pneumoniae infection (82), indicating that γδ T cells are crucial for clearing bacteria during superinfection and that type I IFN production impairs/alters their function. Type I IFN are also known to promote immune effector functions by increasing the expression of perforin and granzyme B in CD8 T and NK cells (83). The role of this downstream cytotoxic effector molecule in the enhanced resistance of IFNAR knockout mice to secondary bacterial pneumonia therefore cannot be excluded.

Interferon gamma, the sole member of type II IFN has been demonstrated to be induced during influenza virus infection, in which CD4+ and CD8+ T cells are the main
producer in lung environment (84). The protective function of this cytokine against influenza virus has been shown to be minimal and it has been suggested to be involved in the susceptibility to secondary bacterial infection (85). Metzger et al. showed that IFN-γ produced during influenza virus infection inhibits subsequent bacterial clearance from the lung. In their study, genetic deletion of IFN-γ and IFN-γ receptor as well as IFN-γ neutralization in the lung conferred resistance to secondary *S. pneumoniae* challenge after influenza virus infection in mice. IFN-γ, induced during primary influenza infection, was found to inhibit phagocytosis of bacteria by macrophages by downregulating expression of scavenger receptor MARCO (72). Recently, Rynda-Apple et al. demonstrated that increased susceptibility of mice to superinfection during influenza infection was associated with IL-13-dependent increased production of IFN-γ (86). In contrast, another study has shown that IFN-γ-deficient and wild-type mice were similarly susceptible to secondary *Staphylococcus aureus* infection after influenza virus infection (81). The use of different strains of bacteria, different doses of influenza and/or bacteria or different timing of infection may explain these contradicting results.

**LCMV and Systemic Secondary Bacterial Infections**

LCMV infection induces generalized enhanced susceptibility to systemic bacterial infection, which has been used to explore the mechanisms of increased predisposition for bacterial infection (87–89). By challenging LCMV-infected mice with *Listeria monocytophages*, *Staphylococcus aureus*, or *Salmonella typhimurium* intravenously, Navarini et al. demonstrated that the virus-induced susceptibility to bacterial invasion was caused by apoptosis of bone marrow granulocytes and impaired granulocyte
emigration to sites of bacterial infection. Although granulocytes were not totally depleted, it became functionally impaired during superinfection to control secondary bacterial infection. Furthermore, they showed that the granulocytopenia-mediated enhanced susceptibility to bacterial infection was facilitated by the induction of type I IFN production after LCMV (89). This mechanism of enhanced susceptibility was also observed in systemic infection of Gram-negative *Pseudomonas aeruginosa*, in which the induction of type I IFN by prior LCMV infection was associated with neutropenia and loss of lysozyme-2-expression in the liver which required to facilitate control of bacteria (87).

The outcome of secondary systemic bacterial infections is also dependent on the time interval between viral and bacterial administration. Gumenscheimer *et al.* demonstrated that the stage of primary infection with LCMV determines the outcome to secondary systemic bacterial infections (88). Mice previously infected with sublethal LCMV showed increased susceptibility at day 3 to Gram-negative *Salmonella typhimurium* or Gram-positive *L. monocytogenes*. However, 8 and 12 days after infection with LCMV mice exhibited a significantly enhanced resistance to systemic infection with either bacterium. The resistance to two completely different bacteria was correlated with the TNF-α hypersensitivity and with the strong, prompt TNF-α and IFN-γ responses to the secondary infection, which are the two cytokines important for resistance to *Salmonella typhimurium* and *L. monocytogenes*. 
**Granzyme B**

**Biology and Cytotoxic Function**

Granzyme B is a serine protease first described as a cytotoxic molecule produced by NK cells and CTL that caused apoptosis in target cells. When CTL and NK cells recognize cell targets, cytotoxic granules which contain granzyme B and perforin move along microtubules to polarize at the plasma membrane near the cell target where they are released into the immunological synapase between CTL/NK cells and cell targets. Perforin alone has been shown to mediate membrane damage, although the combined action of perforin and granzyme B is required for the initiation of apoptosis. In addition to granzyme B, four other different granzymes exist in humans: granzyme A, H, K, and M, while mice also express granzyme A, C–G, K, M, and N. However, granzyme B is the most powerful pro-apoptotic granzyme because of its ability to activate directly and indirectly the target cell’s intrinsic cell death proteases, the caspases (90). On entry into the target cell cytosol, granzyme B promotes apoptosis through two main pathways: BID-dependent mitochondrial permeabilization or direct caspase proteolysis and activation. Proteolysis of BID protein by granzyme B causes BID to translocate to the mitochondria and induce oligomerization of BAX and/or BAK protein in the mitochondrial outer membrane (91, 92). This event disrupts the mitochondrial membrane integrity and increases membrane permeability which facilitates cytochrome c release into the cytosol, assembly of the apoptosome with subsequent caspase 9 activation, and the succeeding caspase 3 and 7 cascade activation. Granzyme B can also directly cleave and activates caspase 3 and 7, hence leads to the degradation of hundreds of cellular caspase protein substrates, which promotes rapid and efficient apoptosis (93). In
addition to the above two major pathways, granzyme B can also directly cleave the inhibitor of a DNase (ICAD) to promote internucleosomal DNA hydrolysis, as well as to cleave a variety of other proteins implicated in the maintenance of nuclear integrity (Lamin B), protection against cell death (MCL-1), DNA repair (DNA-PKcs), microtubule dynamics (α-tubulin), and autoantigens (NuMa, Mi-2) (94).

Granzyme B was previously believed to be only expressed exclusively by NK cells and CTLs, however recent studies in human and mouse have shown that granzyme B can be expressed by various additional cell types (95). In certain pro-inflammatory conditions, granzyme B can be expressed by CD4 T cells, B cells, mast cells, macrophages, neutrophils, basophils, plasmacytoid DCs, T regulatory cells, and nonimmune cell types such as smooth muscle cells (SMCs), chondrocytes, keratinocytes, type II pneumocytes, Sertoli cells, primary spermatocytes, granulosa cells, and syncytial trophoblasts (96, 95), which suggest that in addition to its cytotoxic activity, granzyme B may have non-cytotoxic roles.

**Immunomodulatory Function of Granzyme B**

Granzyme B was largely studied in the context of apoptosis by cytotoxic immune cells as an intracellular protease, however over the past few years, there has been an increased focused on extracellular granzyme B activity. Several studies have shown that granzyme B is present in the serum or plasma, extracellular matrix (ECM) of tissue, and other bodily-derived fluids including synovial fluid and bronchoalveolar lavage (BAL) fluid (96). Under certain conditions that induce cytotoxic immune responses, increased levels of plasma granzyme B has been shown including viral infections (dengue virus,
EBV, CMV, and HIV-1 (97), bacterial infections (*Burkholderia pseudomallei* and *Neisseria meningitides*) (98, 99), as well as during *Plasmodium falciparum* infection (100). In the context of inflammatory vascular disorders including atherosclerosis and transplant vascular disease, granzyme B has been detected in blood vessels, surrounding cells, and extracellular matrix (ECM), in which the level of granzyme B correlates with disease severity (101). Elevated levels of granzyme B are found in the synovial fluid and plasma of rheumatoid arthritis (RA) patients (102), in cerebrospinal fluid of multiple sclerosis patients, as well as in BAL fluid of COPD and hypersensitivity pneumonitis patients (103–105). The presence of increased level of extracellular granzyme B in multiple inflammatory pathologies as described above suggests that this protease may have an important immunomodulatory role.

Recent studies indicate that granzyme B can effectively facilitate or regulate cytokine processing. Interleukin 18 (IL-18), a pro-inflammatory cytokine which plays an important role in systemic and local inflammation, can be cleaved by granzyme B from its inactive to active form (106). Another study demonstrated that IL-1α is a substrate for granzyme B and that proteolysis significantly increased the biological activity of this cytokine *in vitro* and *in vivo*. Granzyme B-deficient mice injected with IL-1α have reduced humoral immune response against ovalbumin antigen compared to wild type mice, suggesting that granzyme B act as an amplifier of inflammatory responses through restricted proteolysis of IL-1α (107). Granzyme B has also been shown to potentiate lipopolysaccharide (LPS)-induced cytokine response of monocytes *in vitro*, a function that also shared with granzyme A and K (108). *In vivo*, granzyme B-deficient mice are protected from lethal LPS challenge compared to wild-type animals (109).
However, Anthony et al. showed that granzyme B knockout mice were not protected from lethal LPS challenge (110). Hence, the effect of granzyme B on the immune response to LPS remains to be resolved. As part of the above immunomodulatory functions of granzyme B, recent study by Prakash et al. showed that granzyme B is used by CTL and NK cells to transmigrate into inflamed tissue (111). By using mouse-models of virus-induced peritonitis, mechanical skin irritation, and cutaneous contact allergy response, they demonstrated that recruitment of CTLs to the site of inflammation is impaired in the absence of granzyme B.

Several proteoglycans including aggrecan, decorin, biglycan, and betaglycan are susceptible to granzyme B-mediated degradation, and these proteoglycans are known to act as a reservoir for cytokines and growth factors such as transforming growth factor-beta 1 (TGF-β1). It was recently shown that cleavage of decorin, biglycan, and beta-glycan by granzyme B liberated active TGF-β1 from the proteoglycans (112). TGF-β1 is known to inhibit the proliferation and effector functions of T cells as well as the activation of macrophages and neutrophils (113, 114). It has also been demonstrated that up-regulation of cellular adhesins by TGF-β, which is activated during influenza viral infection, increases host susceptibility to bacterial co-infection (115).

Furthermore, unpublished observations from our lab suggest that granzyme B has immunosuppressive capacity. We found that DCs isolated from LCMV-infected granzyme B-deficient mice have increased antigen presentation to LCMV transgenic effector T cells. The increased of antigen presentation is independent of perforin, because perforin-granzyme B double knockout mice have even more pronounced antigen presentation compared to either perforin-deficient or granzyme B-deficient mice.
Collectively, it is thus reasonable to speculate that expression of granzyme B may adversely influence the ability of host to mount an effective immune response.

Summary, Hypothesis, and Aims

Influenza and secondary bacterial pneumonia is one of the important health problem in the United States and worldwide. It is well known that following influenza virus infection, bacterial pneumonia such as S. pneumoniae often follows. The mechanisms responsible for this viral-bacterial synergy have remained elusive and several studies using mice have shown that prior influenza virus infection in lung increases bacterial adherence to respiratory epithelium, impairs neutrophil and macrophages recruitment and/or activation, and dysregulates cytokine responses which altogether predispose animals to secondary pneumococcal infection.

Influenza virus infection in the lung has been shown to induce the expression of granzyme B, a serine protease mainly found in cytotoxic lymphocytes which functioned as a cytolytic molecule of immune system. In addition to its cytotoxic activity, there is a growing body of evidence that granzyme B also has non cytotoxic activity such as the cleavage of the extracellular matrix proteins, proteoglycans, which release active TGFβ. Our group have also found that that granzyme B-deficient dendritic cells (DCs) have higher antigen presentation to CD8 T cells in the context of LCMV infection, suggesting that granzyme B has immunosuppressive activity after viral infection. This indicates that granzyme B that is induced during viral infection might predispose host to subsequent infection to other pathogens e.g. bacteria. The capacity of granzyme B to amplify inflammatory response through cytokines processing may also further complicates
pathogenesis of the secondary infections. Hence, we hypothesized that viral-induced granzyme B facilitates the susceptibility to secondary bacterial infection.

![Diagram of hypothetical mechanisms](image)

**Figure 1.** Hypothetical mechanisms of granzyme B role in the susceptibility to secondary bacterial infection

This study was therefore intended to test the above hypothesis by means of two specific aims:

**Specific Aim 1.** Assess the role of granzyme B in the susceptibility to *S. pneumoniae* after influenza virus infection. Working hypothesis: Compared to wild-type mice, granzyme B-deficient mice will be more resistant to secondary *S. pneumoniae* infection following influenza virus infection.

**Specific Aim 2.** Assess the role of granzyme B in the susceptibility to *S. pneumoniae* after LCMV infection. Working hypothesis: Compared to wild-type mice, granzyme B-deficient mice will be more resistant to secondary *S. pneumoniae* infection following LCMV infection.
CHAPTER II Methods and Materials

Mice

All experimental procedures with mice were reviewed and approved by the institutional animal care and use committee (IACUC) at the Cincinnati Children’s Hospital Medical Center. All mice were bred under specific pathogen-free conditions and used on experiment within 8–12 weeks old of age. In all survival study experiments, only female mice were used because previous studies have shown differences in morbidity following infection with influenza virus H1N1 with female mice being more susceptible (116). Wild-type C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). The granzyme B cluster-deficient mice (Gzmb^-/-) were obtained from Dr. Timothy J. Ley and have been previously described (117). In addition to the knockout of the granzyme B gene, these mice are also known to have abrogated expression of several granzyme genes within the granzyme B gene cluster, which include granzyme C, F, D, and G as reported some years later (118, 119).

Cell Culture and Virus Propagation

Madin-Darbin canine kidney (MDCK) cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin G, 100 ug/ml streptomycin (Gibco), 1× glutamax (Gibco), and 25 mM HEPES. HEK293-6E cells were grown and maintained in F17 medium (Gibco) supplemented with 1× glutamax and 1% pluronic F68 (Gibco). Influenza A virus H1N1 A/Puerto Rico/8/34 (PR8) was obtained from Dr. David Woodland and was grown in HEK293-6E cells as previously described. Briefly,
cells were seeded at density of $4 \times 10^6$ cells/ml in F17 complete medium supplemented with 1 ug/ml TPCK-treated trypsin and infected with PR8 at multiplicity of infection (MOI) 0.001. Virus was grown for 48 h at 37°C, 5% CO$_2$ in a static condition. Medium was collected, centrifuged for 15 min at 500 × g, 4°C, to pellet cellular debris, aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. Virus titer was determined using a 50% tissue culture infectious dose (TCID$_{50}$) assay, which determines the dose required to cause cytopathic effect in 50% of the MDCK cells to obtain TCID$_{50}$/ml (120). The virus stocks used in all experiments were from the same batch which were previously passaged three times in HEK293-6e.

LCMV-WE was obtained from Dr. J. de la Torre and grown in BHK-21 cells. Briefly, plate $3.65 \times 10^6$ of cells were plated a day before the infection and infected with LCMV-WE at MOI 0.01. After 24 h, medium was aspirated and replaced with serum-free DMEM and culture was incubated further for 72 h. Medium was collected, centrifuged for 5 min at 500 × g, 4°C, to pellet cellular debris, aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. LCMV titer was determined using plaque assay on Vero cell culture to obtain plaque-forming unit (PFU)/ml.

**Bacterium Propagation**

*Streptococcus pneumoniae* serotype 3 was purchased from American Type Culture Collection (ATCC® 6303™). Glycerol stock of the original culture was first grown in tryptic soy agar (TSA) plate supplemented with 5% sheep blood (Hardy Diagnostics, Santa Maria, CA). Single colony was inoculated into Todd-Hewitt broth with 2% yeast extract supplemented with 5 μl/ml of Oxyrase (Oxyrase, Inc., West
Mansfield, OH) and incubated without shaking at 37°C, 5% CO₂ to late-logarithmic phase as determined by OD₆₀₀ = 0.8–0.9. Bacteria was harvested by adding glycerol to a final concentration 25%, aliquoted, snap-frozen with liquid nitrogen and stored immediately in -80°C. Bacteria titer was determined by spread plating 10-fold serial dilution of the culture on TSA blood plates which were incubated for 24h at 37 °C in 5% CO₂. Colonies of *S. pneumoniae* appeared as grey mucoid colonies surrounded by green zone of alpha-hemolysis when grown in TSA blood plate at above condition. These colonies were counted on plates within the 30–300 CFU range, and multiplied by 10 to obtain CFU/ml. All experiments throughout the study used the same stocks of bacteria.

**Animal Infection**

Influenza virus H1N1 PR8 or *S. pneumoniae* was diluted in sterile PBS to desired concentration and kept in ice before infection. Control mice were given PBS only. Mice were lightly anesthetized with isoflurane and were given infectious agent via oral instillation (oropharyngeal aspiration) as described previously (121). Briefly, mice were placed in a supine position by their upper incisors on surgical thread stretched across a 60° angle incline board. The tongue was gently extended out using sterile pipette tips and 50 µl of infectious agent was instilled at the back of the oral activity above the tracheal opening. The nostrils were then closed while the tongue still extended until fluid aspiration is visually observed followed by several chest retractions. For LCMV-WE infection, mice were infected with 200 PFU of virus intraperitoneally, while systemic
bacterial infection was induced by infecting mice with 10,000 CFU of the bacteria intravenously.

Mice were weighed just before infection and every day after infection. Percentage of body weight loss was calculated as [(weight after infection/weight at day of infection) × 100]. In the survival study, mice which loss greater than 20% of body weight and/or unable to move adequately to eat or drink were considered moribund and euthanized humanely. These mice were included as deceased mice at the time of euthanasia in the survival curve analysis.

**Tissue Collection and Processing**

Mice were sacrificed humanely by CO₂ inhalation or cervical dislocation at desired time points. To collect bronchoalveolar lavage fluid (BALF), chest cavity and trachea were exposed. Catheter needle was inserted into trachea and 1 ml syringe with PBS was loaded to slowly injected into the lungs and aspirated three times. BALF was centrifuged at 500 × g for 5 minutes to pellet the cells, in which supernatant was collected and frozen at -80°C for ELISA while the cell pellet was resuspended in minimal volume for flow cytometry analysis. The left lobe was removed to be used for flow cytometry analysis along with spleen, while the right lobes were collected for viral titer determination. In bacterial titer determination study, the whole lungs were completely removed and used without BALF procedure.

To prepare single cell suspension, lungs were digested with collagenase as previously described (122). Briefly, lungs were minced in enzymatic digestion buffer: 1.8 mg/ml collagenase type 4 (Worthington Biochemical), 0.1 mg/ml DNaseI
(Invitrogen), and 10 mM HEPES in EHAA Click’s medium (Sigma-Aldrich) and incubated at 37°C for 45 minutes in orbital shaker. Enzymatic digestion was stopped with the addition of 5 mM EDTA and samples were subsequently passed through 100 μm cell strainers with syringe plunger to generate single-cell suspensions. Cells were centrifuged for 5 minutes at 500 x g at 4°C and resuspended in flow cytometry buffer (BSS supplemented with 2% FBS and 5 mM sodium azide). Spleens were also processed the same way as lung to obtained DCs with shorter enzymatic digestion incubation time (25 minutes).

**Cell Staining and Flow Cytometry Analysis**

Three million cells from single cell suspensions of individual lung mice were suspended in 100 ul flow cytometry buffer and stained with fluorescently labeled antibodies in the presence on blocking buffer (24.G2 hybridoma culture supernatant). The following monoclonal antibodies against mouse antigens (produced in house or purchased from eBioscience, San Diego, CA; or Biolegend, San Diego, CA) were used: anti-CD45.2 (AL1-4A2), anti-CD4 (GK1.5), anti-CD8α (53-6.72), anti-NK1.1 (PK136), anti-CD11c (N418), anti-MHCII (M5-114), and anti-granzyme B (GB11). Cells were fixed with 2% paraformaldehyde or fixed and permeabilized with FOXP3 Fix/Perm buffer (BioLegend) for intracellular staining. Following 30–60 minutes’ incubation in dark, cells were washed and resuspended in flow cytometry buffer. Flow cytometry data were collected using LSR Fortessa (BD) flow cytometers and analyzed further by FlowJo software (FlowJo, LLC., Ashland, OR).
**ELISA**

Granzyme B concentration from BALF and serum samples were measured by using commercially available sandwich ELISA kit (eBioscience) according to the manufacturer instruction. Briefly, undiluted BALF samples or diluted serum samples (1:10 and 1:30) were incubated for 2 hours at room temperature in 96-well ELISA plates coated with capture antibody. Biotin-conjugated detection antibody was added at incubated for 1 hour at room temperature. Avidin-HRP was added and incubated for 30 minutes at room temperature. 1× TMB substrate was added and incubated for 15 minutes and the reaction was stopped by adding 1 M H₃PO₄ stop solution. Absorbance of the reactions were read on a plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Concentration of granzyme B from the samples were deducted by comparing standard curve which were generated from two-fold serial dilution of recombinant murine granzyme B.

**Lung Homogenization**

For viral titer determination, pre-weighed lung portions were homogenized in DMEM medium (supplemented with 100 U/ml penicillin G, 100 ug/ml streptomycin, 1× glutamax, 0.2% bovine serum albumin, 25 mM HEPES, and 2 ug/ml TPCK-treated trypsin) using a Dounce homogenizer. Lungs were immersed in 1 ml medium followed by 50 and 75 strokes of plunger A and B, respectively. An additional wash of 2 ml medium was added to a final volume of 3 ml. Supernatants were serially diluted for TCID₅₀ analysis in MDCK cell culture grown in 96-well plate to obtain TCID₅₀/g lungs. For bacterial titer determination, lung portions were homogenized in a 500 ul of Todd-
Hewitt broth with 2% yeast extract medium by using plunger A of the Dounce homogenizer. Homogenates were brought up to a 1 ml volume and spread plating of 10-fold serial dilution on TSA blood plates was used to determine CFU/lungs.

**Statistical Analysis**

All statistical comparisons were performed using GraphPad Prism 6. Quantitative data differences between two groups were compared by the unpaired Student’s T test and Mann-Whitney test for populations with normal and non-normal distribution, respectively. Survival curves between two groups were compared by Mantel-Cox logrank test. $P$ value less than 0.05 was considered significant.
CHAPTER III RESULTS

Dose-dependent Weight Loss and Mortality in Influenza-infected Wild-type C57BL/6J Mice

To allow the assessment of granzyme B role in the susceptibility to secondary bacterial infection after influenza virus challenge, we first performed dose titration experiments to find the dose of influenza virus that caused no mortality in WT mice. We infected groups of age-matched female WT mice with 300, 1,000, 3,000, 30,000, and 100,000 TCID$_{50}$ of influenza virus PR8 via oral instillation. Morbidity and mortality were assessed by recording the weight loss which has been used as a primary objective measure of the severity of influenza virus infection as well as a good way to track disease progression in mice (123).

As shown in Figure 2, infection with a dose of 300, 1,000, and 3,000 TCID$_{50}$ induced transient weight loss that peaked at day 8. However, a dose of 30,000 and 100,000 TCID$_{50}$ caused excessive morbidity and mortality in which most of the mice lost more than 20% of their original body weight and eventually succumbed to infection. The median survival for a dose of 30,000 and 100,000 TCID$_{50}$ was 5.5 and 5 days, respectively based on Kaplan-Meier survival curves analysis. No mortality was observed in mice infected with a dose of 3,000 TCID$_{50}$ or below, while oral instillation challenge with PBS only did not cause any apparent morbidity. Mice infected with 300, 1,000, and 3,000 TCID$_{50}$ loss weight up to 3.1, 4.8, and 13.2% of its body weight, respectively. A dose of 1,000 TCID$_{50}$ influenza virus PR8 was, therefore, selected for subsequent experiments as this sublethal dose was able to induce apparent moderate morbidity with no mortality observed.
Figure 2. Dose-dependent weight loss and mortality in influenza-infected wild-type C57BL/6J mice. Groups of female wild-type mice were infected with indicated doses of influenza virus PR8 or PBS. Weights from each mouse were recorded before infection (day 0) and on a daily basis following infection through the end of the study. Change in percentage of starting weight was calculated for each mouse and plotted (left panel). Survival of mice was monitored and Kaplan-Meier survival curves were plotted (right panel). Data are pool of three experiments.

Sublethal Influenza Virus Infection Induces Expression of Granzyme B in Pulmonary CD8, CD4, NK, and DC

To characterize influenza virus infection in WT mice in the context of granzyme B induction, we analyzed the expression of pulmonary intracellular expression of granzyme B during the course of sublethal influenza virus infection. Although granzyme B has been reported to be predominantly expressed by CD8 T cells and NK cells, several studies and findings from our laboratory have found that murine CD4 T cells (Treg and cytolytic CD4 cells) and DCs expressed granzyme B in the context of viral infection (25). To identify granzyme B-expressing cells in the lung during influenza infection, lung and BALF cells were isolated at day 3, 7, 10, and 14 after influenza virus infection and stained for cell surface markers and intracellular granzyme B which then analyzed by flow cytometry (Figure 3A and 3C). As shown in Figure 3B, CD8 T cells and NK cells were the predominant cells that produce granzyme B with 2.8-log and 1.8-log increase of granzyme B-positive cells, from baseline level (uninfected) to the peak of response at day 7. A limited number of CD4 T cells and DCs positive for granzyme B
were observed following influenza virus infection. By day 14 post-influenza virus infection, the number of granzyme B-positive cells have returned to baseline uninfected level in all subset of cells. Similar trends were also observed in cells isolated from BALF (Figure 3B and 3D), however with lesser fold-increase of granzyme B-positive cells from baseline to the peak of production at day 7 (2.1-log, 0.6-log, 1.7-log, 1.1-log increase for CD8, CD4, NK, and DCs, respectively).

Figure 3. Sublethal influenza virus infection induces expression of granzyme B in pulmonary CD8 T cells, CD4 T cells, NK cells, and DCs. Wild-type mice were infected with 1,000 TCID<sub>50</sub> influenza virus and sacrificed at indicated time points (n = 4 mice/time point, or 2 mice at “ naïve”). Representative plots of whole lungs (A) and BALF (B) cells for intracellular
granzyme B staining in CD8 T cells (autofluorescence\textsuperscript{lo}CD45.2\textsuperscript{+}CD8\textalpha\textsuperscript{+}), CD4 (autofluorescence\textsuperscript{lo}CD45.2\textsuperscript{+}CD4\textsuperscript{+}), NK cells (autofluorescence\textsuperscript{lo}CD45.2\textsuperscript{+}NK1.1\textsuperscript{+}), and DCs (autofluorescence\textsuperscript{lo}CD45.2\textsuperscript{+}CD8\textalpha\textsuperscript{–}MHC II\textsuperscript{+}CD11c\textsuperscript{+}) from individual mouse. Autofluorescence was defined by distinguishable positive signal from open FITC channel. Number shown in the plots are percentage of granzyme B positive cells from parent gate.
Quantitative analysis of absolute number of whole lungs (C) and BALF (D) cells of granzyme B positive cells from indicated cell population shown in (A) and (B). Data are shown as mean ± SEM with n = 4 mice/time point, or 2 mice at “naïve”.

Sublethal Influenza Virus Infection Induces Expression of Extracellular Granzyme B in Pulmonary Airways

Given that flow cytometry analysis indicated that specific cell types expressed granzyme B, we then performed ELISA for granzyme B from BALF of influenza infected mice. Extracellular granzyme B expression, as measured by ELISA, was transiently increased and peaked by 3-log increase at day 7 after infection and returned to baseline by day 14 (Figure 4). Mean extracellular granzyme B concentration from 6–8 mice were 10.2 ± 6.3, 2,413.0 ± 514.5, 176.1 ± 55.3, 2.0 ± 1.0 pg/ml at day 3, 7, 10, and 14, respectively with no detectable granzyme B in uninfected mice. Moreover, granzyme B expression in serum from these influenza-infected WT mice were not detectable. Indicating a localized granzyme B expression in the lungs.
Figure 4. Sublethal influenza virus infection induces expression of extracellular granzyme B in pulmonary airways. Wild-type mice were infected with 1,000 TCID$_{50}$ influenza virus and bronchoalveolar lavage fluid (BALF) were collected for granzyme B measurement by ELISA at day 3, 7, 10, and 14 after influenza virus infection. Dot plot from individual mice are shown and the mean shown as a horizontal bar with SEM. Undetectable samples were assigned a value of 1 pg/ml for the purpose of analysis. Data are from two independent experiments with four to eight mice at each time point.

Similar Body Weight Loss and Viral Load of Wild-type and Granzyme B-deficient Mice During Influenza Virus Infection

As the main objective of this study was to investigate the role of granzyme B in secondary bacterial infection following influenza virus infection, we first investigated the contribution of granzyme B to the host response during primary influenza virus infection. Wild-type and $Gzmb^{-/-}$ mice were infected with 1,000 TCID$_{50}$ influenza virus, in which morbidity as reflected by body weights changes and viral loads were analyzed at indicated time points during the course of infection (Figure 5). Transient body weight loss between WT and $Gzmb^{-/-}$ mice were similar throughout the course of infection, which were most pronounced at day 8 (4.8 ± 1.2% and 4.0 ± 1.2% decreased of body weight in WT and $Gzmb^{-/-}$, respectively). Both group of mice had completely returned to its original body weight by day 11. Likewise, viral loads which measured at day 3, 7, and 14 after influenza virus infection were similar in both mouse strains with no
detectable virus in the lungs at day 10 and 14. The similarity of both body weight loss and viral load between WT and Gzmb\textsuperscript{−/−} mice excluded the potential baseline differences prior to infection with \textit{S. pneumoniae}.

Figure 5. Similar body weight loss and viral load of wild-type and granzyme B-deficient mice during influenza virus infection. Female wild-type and Gzmb\textsuperscript{−/−} mice were infected with 1,000 TCID\textsubscript{50} of influenza virus PR8. (A) Body weight changes during the course of infection were recorded. Values are means ± SEM and are representative of four to five independent experiments with four to eight mice per group. (B) Viral load in the lungs were analysed at day 3, 7, 10, and 14 after influenza virus infection. Data shown are the mean ± SEM titers of three mice at each time point from each group.

**Dose- and Time-dependent Mortality in Secondary \textit{S. pneumoniae} Infection Post-influenza Virus Pneumonia**

To establish a model for secondary bacterial infection post-influenza virus infection, we conducted a bacterial dose response analysis to find doses of \textit{S. pneumoniae} that are at least 50\% lethal if were given to influenza-infected animals but are not lethal to naïve wild-type mice. We infected WT mice with 300, 1,000, or 10,000 CFU of \textit{S. pneumoniae} or with 1,000 TCID\textsubscript{50} influenza virus followed by infection with 300, 1,000, or 10,000 CFU of \textit{S. pneumoniae} on day 14 and assessed the morbidity by body weight loss as well as mortality. As shown in Figure 6, single infection with 300, 1,000, and 10,000 CFU of \textit{S. pneumoniae} caused 0, 25, and 100\% mortality, while the same doses of \textit{S. pneumoniae} in influenza-infected mice caused 57, 75, and 100\%
mortality, respectively. Furthermore, when we tested the combination of 1,000 TCID$_{50}$ influenza virus followed by 300 CFU S. pneumoniae infection at day 7 and 10, mortality was increased to 100 and 90%, respectively. Together these results demonstrate increases susceptibility to secondary bacterial infection following primary influenza virus infection.

Figure 6. Time- and dose-dependent mortality in secondary S. pneumoniae infection post-influenza virus pneumonia. (A) Groups of female wild-type mice were infected with 300, 1,000, or 10,000 CFU of S. pneumoniae or with 1,000 TCID$_{50}$ influenza virus followed by infection with 300, 1,000, or 10,000 CFU of S. pneumoniae on day 14. (B) Mice were infected with the same dose of influenza virus followed by 300 CFU S. pneumoniae at day 7, 10, or 14. Mortalities were recorded and data shown as percentage of survived mice.
Wild-type and Granzyme B-deficient Mice Are Both Susceptible to Secondary S. pneumoniae Infection After 10 Days of influenza Virus Infection

To assess the role of granzyme B in the susceptibility to secondary bacterial infection, we first performed experiments where we infected WT and Gzmb−/− mice with 1,000 TCID₅₀ influenza virus or PBS (mock) followed by 300 CFU S. pneumoniae at day 10. While no apparent morbidity and mortality seen in PBS+S. pneumoniae-infected animals from both WT and Gzmb−/− group, secondary bacterial infection caused 89% and 80% mortality in WT and Gzmb−/− mice previously infected with influenza virus (Figure 7). The median survival time in which 50% of the mice were still alive in both WT and Gzmb−/− was 14 days (or 4 days after secondary infection). The same trend was also observed when we infected mice with lower dose of S. pneumoniae (30 CFU) with the same dose of influenza virus and time interval between the two infectious agents. Both group of animals were succumbed to secondary infection with 67% and 50% mortality observed in WT and Gzmb−/− group, respectively. The median survival time in this lower bacterial dose challenge was 14.5 (WT) and 15.5 days (Gzmb−/−) and Kaplan-Meier survival curve analysis showed no significant difference between the two groups. These results suggest that granzyme B is not required for enhanced susceptibility to secondary bacterial infection after 10 days of primary influenza virus infection.
Figure 7. Wild-type and granzyme B-deficient mice are both susceptible to secondary *S. pneumoniae* infection after 10 days of influenza virus infection. Female wild-type and Gzmb$^{-/-}$ mice were infected with 1,000 TCID$_{50}$ of influenza virus PR8 followed by 300 CFU (upper panel) or 30 CFU (lower panel) *S. pneumoniae* at day 10. Daily body weight changes during the course of infection (left panel) and mortality (right panel) were recorded. Values shown at the left panels are means ± SEM.
Wild-type and Granzyme B-deficient Mice Are Both Susceptible to Secondary *S. pneumoniae* Infection After 14 Days of Influenza Virus Infection

To further test whether the similar susceptibility between WT and *Gzmb*−/− to secondary bacterial infection was caused by the timing of administration of the two agents, we performed experiments in which mice were infected with influenza virus and *S. pneumoniae* 14 days apart. This timing would eliminate any influence of residual influenza in the lungs of infected mice that could potentially cause excessive mortality during secondary bacterial challenge. Furthermore, Ramphal *et al.* showed that the respiratory airways of influenza-infected mice undergone regeneration that requires 14 days for complete repair (124). Hence, any damage associated with the influenza virus infection could be excluded when infecting mice with *S. pneumoniae* 14 days apart. WT and *Gzmb*−/− mice were infected with 1,000 TCID₅₀ influenza virus PBS (mock) followed by 300 CFU *S. pneumoniae* infection at day 14. Similar to the day 10 experiments, 73.3% WT and 87.5% *Gzmb*−/− mice died after secondary bacterial challenge with median survival time 19 and 18 days for WT and *Gzmb*−/− group, respectively (Figure 8). When mice were infected with lower bacterial dose (30 CFU) at day 14, all of the *Gzmb*−/− survived while 29% of WT mice died. However, this difference of mortality was not significant based on the Kaplan-Meier survival curve analysis. These results suggest that granzyme B has a minimal role during secondary bacterial infection even after 14 days of primary influenza virus infection.
Figure 8. Wild-type and granzyme B-deficient mice are both susceptible to secondary \textit{S. pneumoniae} infection after 14 days of influenza virus infection. Female wild-type and \textit{Gzmb}^{−/−} mice were infected with 1,000 TCID$_{50}$ of influenza virus PR8 followed by 300 CFU (upper panel) or 30 CFU (lower panel) \textit{S. pneumoniae} at day 14. Daily body weight changes during the course of infection (left panel) and mortality (right panel) were recorded. Values shown at the left panels are means ± SEM.

Similar Bacterial Burden in Wild-Type and Granzyme B-deficient Mice After Secondary Infection Post-Influenza Virus Pneumonia

To further determine whether the susceptibility of both WT and \textit{Gzmb}^{−/−} to secondary bacterial infection was caused by the impaired bacterial clearance from the lungs, we infected both group of mice with 1,000 TCID$_{50}$ influenza virus or PBS (mock) followed by 30 CFU \textit{S. pneumoniae} infection 10 days later. Forty-eight hours post secondary bacterial infection, mice were sacrificed and lung bacterial load was quantified. Wild-type and \textit{Gzmb}^{−/−} mice infected only with \textit{S. pneumoniae} have
controlled bacterial growth (mean titer WT 477.5 and \( Gzmb^{-/-} \) 106.7 CFU/lung) without significant difference between the two groups, while prior influenza virus infection increased bacterial burden by 5 logs and 6 logs in WT and \( Gzmb^{-/-} \) mice, respectively (Figure 9). The difference in bacterial titer between post-influenza WT and \( Gzmb^{-/-} \) mice was not statistically different, suggesting a minimal role of granzyme B in impairing bacterial clearance in the context of post-influenza virus infection.

![Graph showing bacterial load comparison between WT and Gzmb^{-/-} mice after secondary infection post-influenza virus pneumonia](image)

**Figure 9.** Similar bacterial burden in wild-type and granzyme B-deficient mice after secondary infection post-influenza virus pneumonia. Female wild-type and \( Gzmb^{-/-} \) mice were infected with 1,000 TCID\(_{50}\) of influenza virus PR8 followed by 30 CFU of \( S. \) pneumoniae 10 days later. Dot plot from individual mice are shown and the mean shown as a horizontal bar with standard error mean. ns, not significant.

**Wild-type and Granzyme B-deficient Mice Are Both Susceptible to Systemic \( S. \) pneumoniae infection 10 days after LCMV infection**

As we previously found that granzyme B-deficient mice have increased T cells activation and DCs antigen presentation independent of perforin in LCMV infection model, we further extended our investigation in this model to examined whether granzyme B deficiency protects from secondary bacterial infection post-LCMV infection.
We first performed experiments to assess the kinetic of extracellular serum granzyme B in WT mice infected with LCMV. We infected WT mice with 200 PFU of LCMV-WE and serially measured the granzyme B concentration from blood serum. Granzyme B level increased after LCMV infection at day 6 and peaked to almost 4 log at day 8 before declining at day 10 (Figure 10).

![Graph of Serum Granzyme B](image)

**Figure 10.** LCMV infection induces expression of serum granzyme B. Wild-type mice were infected with 200 PFU LCMV-WE and blood serum were collected for granzyme B measurement by ELISA at day 3, 6, 8, 10, 13, and 15. Data shown are the mean ± SEM of 3–24 mice at each time point.

We then conducted experiments in which we infected WT and Gzmb<sup>−/−</sup> mice with 200 PFU LCMV followed by intravenous infection of 10,000 CFU *S. pneumoniae* at day 10. This time point was chosen because another study has shown that infectious virus is undetectable in spleens and livers of WT and Gzmb<sup>−/−</sup> mice by 10 days after LCMV infection (125). Hence, although we did not characterize the LCMV viral load in our experiments, there is little evidence to suggest that LCMV might directly interact with *S. pneumoniae*. LCMV is also generally non-cytopathic in its murine host, therefore direct cell destruction by replicating virus may not contribute to the enhancement of bacterial
superinfection. LCMV infection in these two group of mice caused transient weight loss at day 8–9 in which mice quickly regained weight afterwards. Furthermore, LCMV infection alone was not lethal in either group even after 3 weeks of infection (data not shown). Naïve WT and Gzmb$^{-/-}$ mice were also resistant to intravenous challenge of 10,000 CFU S. pneumoniae (Figure 11). However, when LCMV-infected mice were challenged with 10,000 CFU S. pneumoniae, 75.0% WT and 85.7% Gzmb$^{-/-}$ mice died with median survival time 13 and 12.5 days, respectively. The difference of mortality between post-LCMV WT and Gzmb$^{-/-}$ mice was not statistically different based on the Kaplan-Meier survival curve analysis, suggesting once again a minimal role of granzyme B in impairing bacterial clearance post-viral infection.

Figure 11. Wild-type and granzyme B-deficient mice are both susceptible to secondary systemic S. pneumoniae infection after 10 days of LCMV infection. Female wild-type and Gzmb$^{-/-}$ mice were intraperitoneally infected with 200 PFU LCMV-WE or PBS (mock) followed by 10,000 CFU S. pneumoniae at day 10. Daily body weight changes during the course of infection (left panel) and mortality (right panel) were recorded. Values shown at the left panels are means ± SEM.
CHAPTER IV DISCUSSION

Secondary bacterial infections have been shown to be frequently associated with influenza related complications and death. A number of studies have uncovered important mechanisms by which prior influenza virus infection predisposes host to uncontrolled secondary bacterial infection, including increased bacterial adherence to respiratory epithelium, impaired neutrophil and macrophages recruitment and/or activation, and dysregulated cytokine responses. Here, we tested the hypothesis that granzyme B contributes to the susceptibility to secondary bacterial infection following influenza virus infection. This hypothesis was based on the following lines of evidence: 1) granzyme B suppress antigen presentation by DCs independent of perforin (unpublished observation); 2) granzyme B liberates immunosuppressive TGF-β from proteoglycans (112); 3) granzyme B degrades a number extracellular matrix (101, 126, 127); and 4) granzyme B expression is increased in CTLs upon infection with influenza virus (128). However, we rejected this hypothesis as we found that granzyme B was not required for increased susceptibility to secondary bacterial infection in two independent viral infection model.

Sublethal influenza virus infection in our study induced production of extracellular granzyme B in pulmonary airway space and intracellular expression in pulmonary lymphocytes (CD8, CD4, NK), and DCs. Although the majority of granzyme B is released towards target cells with the aid of perforin, granzyme B can also be released nonspecifically, escaping into the extracellular milieu (127, 129). The mechanisms by which granzyme B released extracellularly have been proposed, including incidental escape from immunological synapse during or after target cell killing, constitutive
granzyme secretion by CTLs after degranulation, and induction of granzyme release from CTLs by extracellular stimuli (127). In addition, many other cell types that express granzyme B do not co-express perforin which allows non-specific release of granzyme B and accumulation in the extracellular space (130). Several studies have demonstrated increased level of active extracellular granzyme B from BALF samples of patients infected with respiratory syncytial virus (RSV) (131), hypersensitivity pneumonitis (105), allergen-challenged atopic asthma (132), and in COPD patient (104), suggesting that extracellular granzyme B in the lungs is involved in the pathogenic inflammation response.

Our initial experiments showed that granzyme B is not essential in the host response to influenza virus infection alone to a significant degree. We found that the transient body weight loss and the kinetic of viral load burden were similar in Gzmb⁻/⁻ mice when compared with normal WT mice. Indeed, Jenkins et al. showed that mice deficient in granzyme A and B do not show increased susceptibility and can control influenza virus infection as effectively as WT mice (133). Moreover, influenza virus-infected Gzmb⁻/⁻ mice displayed a similar in vivo cytotoxic competency when compared with influenza virus-infected WT mice (134). Similarly, lungs viral burden from pneumovirus-infected WT mice were indistinguishable from those measured in lungs from pneumovirus-infected Gzmb⁻/⁻ mice (135). Granzyme B, however, is important for controlling murine cytomegalovirus (MCMV) replication and ectromelia poxvirus, as mice deficient in granzyme B are unable to control primary infection by these pathogens (136, 137). This suggests that granzyme B may have differential roles which depend on the primary site of infection and/or the causative pathogen.
Our study also showed that both WT and \( Gzmb^{-/-} \) mice had already eliminated influenza virus at day 10 post-infection. This allowed us to challenge mice with \( S. \) pneumoniae at day 10–14 to avoid excessive pulmonary airways damage left from primary influenza virus infection as well as to exclude a direct interaction between influenza virus and \( S. \) pneumoniae in the lungs. Clinical data from epidemiological study indicates that 7–15 days is a common interval window between influenza infection and the occurrence of secondary bacterial complications (138, 139). Most studies of secondary bacterial infection however infected mice within 3 to 7 days' post-influenza virus infection and thus, findings from these studies should be interpreted very carefully as these might not reflect the real clinical situation in humans. The presence of viral replication during early time points in such studies might also prohibited the exclusion of direct interaction between influenza virus and \( S. \) pneumoniae in the pulmonary environments.

We recognized that day 10 and 14 post influenza virus infection may not be relevant to the peak of granzyme B induction since at these time points granzyme B level has already decreased. However, Hirota et al. have demonstrated that, using bleomycin-induced acute lung injury, \( Gzmb^{-/-} \) mice developed a significant increase of fibronectin 21 days after bleomycin exposure compared to WT mice which was associated with a greater magnitude and duration of lymphocyte-dominated inflammation suggesting a long term indirect effect of granzyme B in regulating immune response (140). Furthermore, Didierlaurent et al. showed a long-term alterations of an innate immune pathway after resolution of respiratory viral infection. Although they suggested that the increased susceptibility to secondary bacterial infection even after 4–
6 weeks post-influenza virus infection is attributed to desensitization of macrophages to TLR signals, the involvement of CD8 T cells, the major producer of granzyme B, cannot be excluded entirely as a population of these cells persist in the lung airways 8 weeks after influenza virus infection (141).

Our experiments in WT mice further indicate that even when given 10 to 14 days apart, prior influenza virus infection enhances susceptibility of mice to succumb by low dose of *S. pneumoniae* challenge, with the peak of lethality occurred when the two agents were administered 7 days apart. With only 300 CFU *S. pneumoniae* which was non-lethal to naïve mice, we were able to observe enhanced mortality in influenza-infected mice. Surprisingly, almost all of the studies of secondary bacterial pneumonia in animal models used high doses of pneumococcus bacteria ($10^3$–$10^9$ CFU) which can lead to overwhelming bacteria growth in the lungs and highly lethal bacteremia (142). Using a relatively small dose of pneumococcus (300 CFU) is perhaps more physiologically relevant to the clinical setting of secondary bacterial pneumonia.

As the WT and *Gzmb*−/− mice were both responded to primary influenza virus infection in similar fashion, the role of granzyme B in the host response to post-influenza bacterial pneumonia can be feasibly evaluated. In the present study, we found that granzyme B deficiency does not prevent the enhanced susceptibility of secondary bacterial infection post-influenza virus infection, which is demonstrated by following findings: (1) similar susceptibility of WT and *Gzmb*−/− mice to mortality caused by secondary *S. pneumoniae* infection after 14 and 10 days of influenza virus infection; and (2) similar bacterial burden in influenza virus-infected WT and *Gzmb*−/− mice. The present findings here oppose our previous observations in which *Gzmb*−/− mice have
increased ex vivo DCs antigen presentation after acute viral infection suggesting that granzyme B is immunosuppressive to DCs in the context of post-viral infection.

DCs together with macrophages are the early phagocytic cells that take up and kill bacteria in addition to coordinating the innate and adaptive immune response to S. pneumoniae, although neutrophils are the most important cells for the clearance of S. pneumoniae (47). Moreover, Colino et al. demonstrated that DCs actively uptake and present both polysaccharide and protein antigens expressed by intact extracellular S. pneumoniae to promote humoral immune response in vivo (143). One study has also shown the important role of respiratory DCs to activate invariant natural killer T (iNKT) cells which stimulate anti-streptococcal effects through neutrophils (144, 145).

Interestingly, Rosendahl et al. demonstrated that DC-depleted mice exhibited delayed bacterial systemic dissemination and significantly reduced bacterial loads after intranasal challenge with S. pneumoniae than non-depleted animals, which was associated with a better capacity of DCs-depleted mice to restrict systemic pneumococci dissemination (146). The similar susceptibility to secondary bacterial infection between WT and Gzmb−/− mice implies that immunosuppression to DCs may not directly involved to the increased of host susceptibility to secondary bacterial infection. Further studies are required to establish the definitive role of DCs in the context of secondary bacterial infection post-influenza virus. It would also be noteworthy to assess whether DCs antigen presentation is increased in the absence of granzyme B after influenza virus infection as we saw in LCMV infection.

While uncontrolled bacterial burden may be the major cause of disease pathogenesis in secondary bacterial infection after influenza virus infection,
immunopathology as a result of excess inflammation is commonly observed and implicated. In the majority of clinical cases during influenza pandemics, autopsy lung samples displayed immunopathological changes that include massive infiltration of neutrophils (5, 147). Massive ‘cytokine storms’ of TNF-α, IL-1β, IL-6, IL-18, CXCL1 (KC), CXCL2 (MIP-2), CCL3 (MIP-1α) and the consequential inflammatory cell infiltration have been observed in a number of influenza/bacterial coinfection animal models (148–150). As discussed before, granzyme B has been demonstrated to be able to increase the activity of IL-18 and IL-1α which can amplify the downstream inflammatory response (106, 107). It is therefore possible that the induction of granzyme B during influenza virus infection triggers excess inflammation which can complicate the pathogenesis of secondary bacterial infection. Although we did not see any difference in terms of mortality and bacterial burden between WT and Gzmb−/− mice during secondary bacterial infection, granzyme B-deficient mice might benefit from having less inflammatory response compared to granzyme B-competent mice. Further studies are therefore needed to evaluate this proposition.

Influenza virus infection induced localized granzyme B expression in the lungs without detectable level in the serum even at the peak of induction in the lungs (day 7). In contrast, LCMV infection induced serum granzyme B expression to almost 4 log increase from baseline uninfected level (from <40pg/ml to 7094.1 ± 1113.5 pg/ml) at day 8 after LCMV infection. However, similar to what we saw in influenza-infected mice, mortality by secondary intravenous S. pneumonieae challenge post-LCMV infection is similar between WT and Gzmb−/− mice, suggesting a dispensable role of granzyme B in systemic secondary infection of S. pneumonieae.
Encapsulated bacteria such as *S. pneumoniae* entering the blood-stream has been shown to be removed by phagocytic cells mostly macrophages in the liver and spleen (151, 152). DCs have also been demonstrated to indirectly contribute to systemic bacterial clearance by their IL-12 production, rather than direct bacterial uptake and killing (153). However, the enhanced *ex vivo* antigen presentation by Gzmb−/− DCs which were observed in our previous experiments may not be necessary for resistant against systemic secondary bacterial infection. It is also unlikely that LCMV-induced bone marrow granulocytopenia involved in enhanced susceptibility to systemic bacterial infection as shown by Navarini *et al.* (89), since we infected mice 10 days post-LCMV infection, the time that bone marrow granulocyte number has already returned back to uninfected baseline level.

The absence of protection in granzyme B-deficient mice from secondary bacterial infections suggest that other mechanisms might play greater role in predisposing host following primary viral infection. It is also possible that compensatory mechanism by other granzymes such as granzyme A, K and M may exist. Granzyme A and K has been shown to modulate pro-inflammatory cytokines response, such as IL-1β, TNF-α, IL-6, and IL-8 (154), while granzyme A- and M-deficient mice are more resistant to lethal inflammatory response caused by LPS challenge (110). However, CD8 T cells from LCMV-infected granzyme A- or B-deficient mice contained similar level of granzyme K and undetectable level of granzyme M (155), which is also confirmed in granzyme-A- and B double knockout mice infected with influenza virus (133). Thus, the importance of these granzymes in impairing the immune response to secondary bacterial infection following viral infection need to be further studied.
CHAPTER V CONCLUSIONS AND FUTURE DIRECTIONS

It has been well recognized that sublethal influenza virus infection predispose the host to be more susceptible to secondary bacterial infection with \textit{S. pneumoniae}, which has been shown by our present study and others. Our study collectively demonstrated that extracellular and intracellular granzyme B, which is highly expressed in the lung during influenza virus infection, plays a minimal role in the enhanced susceptibility to secondary \textit{S. pneumoniae} infection following influenza virus infection. Similarly, expression of serum granzyme B during LCMV infection is not required to the increased susceptibility to systemic \textit{S. pneumoniae} challenge post-LCMV infection.

Further studies are needed to ascertain the role of other granzymes in the mechanisms of these concomitant viral and bacterial infections. Granzyme A have been shown to be induced during influenza virus infection by our lab (unpublished observation) as well as others (128, 156). The ability of granzyme A to induce a number of proinflammatory cytokines may have a deleterious impact during secondary bacterial infection rather than protection (154, 157). Further investigations by using \textit{Gzma}^{-/-} and \textit{Gzma}^{-/-}\textit{Gzmb}^{-/-} double knockout mice may help elucidate the role of this granzyme to increase susceptibility to secondary bacterial infections.

Granzyme B- and A-deficient mice have been shown to be resistant to lethal challenge of LPS, which is the major outer surface membrane components present in Gram-negative bacteria such as \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, and \textit{Haemophilus influenzae} (158). Therefore, it would be interesting to see whether granzyme B- and/or A-deficiency protect mice from secondary infections by these Gram-negative pathogens as they have also been found to be associated with several...
influenza virus pandemics (5, 159). Furthermore, it may also be important to see whether granzyme B deficiency lead to resistant to secondary bacterial infections after infection by less pathogenic influenza virus strain such as X31 which has the HA and NA genes of A/Hong Kong/1/1968 (H3N2) in the background of PR8 strain (160). Compared to PR8 strain used in our study, the X31 strain induces less lung tissue damage which associated with lower viral growth and early activation of TREM1 and IL-17 signaling pathways (161). Additionally, Peltola et al. have showed that strain-specific level of influenza virus neuraminidase activity correlated with their differential ability to support secondary bacterial pneumonia (67). Hence, it would be of great interest to use different combination of pathogens to investigate the role of granzyme B as well as granzyme A in increasing host susceptibility to secondary bacterial infection.

Ultimately, the challenge remains to investigate the immunomodulatory role of granzyme B in the context of post-viral infection and these future directions will partly contribute to advance our understanding in the complex mechanism of bacterial-viral coinfection.
REFERENCES


accumulation in influenza-infected mice. Am. J. Pathol. 85: 373–82.


