DISEASE INTERVENTION STRATEGIES AGAINST RESPIRATORY SYNCYTIAL VIRUS INFECTION

by

WENLIANG ZHANG

(Under the Direction of Ralph A. Tripp)

ABSTRACT

Respiratory syncytial virus (RSV) is a major cause of morbidity and some mortality in infants, young children, and the elderly worldwide. Currently, there is no effective vaccine. The antiviral drugs to control RSV infection are limited. The increasing disease burden and slow progress toward vaccine development is driving the search for new disease intervention strategies against RSV infection. RNA interference (RNAi) is a powerful tool amenable for the development of antiviral drugs. siRNAs targeting the RSV P gene (siRNA-P) have been shown to silence RSV replication both in vitro and in a BALB/c model of RSV infection. In this study, we examined the effect of siRNA-P prophylaxis on the primary and memory immune response to RSV infection in BALB/c mice. The central hypothesis of the study was siRNA-P could be used to reduce RSV replication to a level that did not cause disease pathogenesis but still allowed for robust immunity to infection. We show that mice prophylactically treated with siRNA-P to decrease but not to eliminate RSV replication exhibit reduced pulmonary inflammation and lung

pathology, and produce an effective anti-RSV memory response when subsequently challenged with RSV. The results suggest that siRNA can be developed as an effective antiviral drug that can be used to reduce the viral load and parameters of pathogenesis without limiting the induction of the memory immune response.

RSV surface proteins have been shown to modulate the host immune response to infection, an effect that has hindered vaccine development. The RSV G protein has been shown to contribute to the majority of immune modulation. A unique feature of the RSV G protein is that its central conserved region contains a CX3C chemokine motif, which has been shown to mimic the activities of the only known CX3C chemokine, fractalkine, and bind to the fractalkine receptor, CX3CR1, a feature that has been shown to modulate the immune response and cause disease pathogenesis in mice. In this study, we tested the hypothesis that G protein peptides and/or polypeptides could induce antibodies that blocked G protein interaction with CX3CR1. The results showed that antibodies specific for the CX3C motif had high blocking activity. These results were confirmed in mice vaccinated with G protein peptides and polypeptides where recovered antisera were tested for blocking antibodies and pulmonary disease pathogenesis was evaluated. These results suggest that vaccines can be made which induce G protein CX3C-CX3CR1 blocking antibodies, and that this vaccine strategy may be useful to prevent G protein immune modulation and disease pathogenesis.

INDEX WORDS: Respiratory syncytial virus, Small interfering RNA, Vaccine, Chemokine, Disease intevention

DISEASE INTERVENTION STRATEGIES AGAINST RESPIRATORY SYNCYTIAL VIRUS INFECTION

by

WENLIANG ZHANG

B.S. Ningbo University, P.R. China, 2000

MMED, Shanghai Jiaotong University, P.R. China, 2003

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

DISEASE INTERVENTION STRATEGIES AGAINST RESPIRATORY SYNCYTIAL VIRUS INFECTION

by

WENLIANG ZHANG

Major Professor: Ralph A. Tripp

Committee: Jeff Hogan
Mark Tompkins
Kim Klonowski
Zhen Fu

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009

DEDICATION

This dissertation is dedicated to my wife and many friends, without whom I could never have come so far.

ACKNOWLEDGEMENTS

There are many people I would like to thank. Most importantly, I would like to thank my advisor, Dr. Ralph Tripp for his thorough guidance and great leadership throughout my PhD career. Without his continuous support, it would have been impossible to reach this. I would like to thank my committee members, Dr. Mark Tompkins, Dr. Jeff Hogan, Dr. Kim Klonowski and Dr. Zhen Fu for their helpful suggestions and valuable feedback. I would also like to thank Les Jones for his assistance in protein purification and advice, and Jamie Barber for his help and advice on flow cytometry. A special thanks goes to Christine Oshansky, my labmate and friend, for her helpful discussion, suggestions and support during my stressful times. I would also like to thank Stephanie Gavrielides, our previous department secretary, for her patience and help. I would like to thank all the people in animal health research center (AHRC) for their great support.

Lastly, I thank my wife, Yi Kuang for her incredible support and patience in both my studies and life over the past few years. I could not have done it without her.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Respiratory syncytial virus (RSV) is the major cause of serious lower respiratory tract disease in infants, young children, the elderly and the immune suppressed worldwide (1, 4, 14, 18, 19, 22, 27, 46, 48). Since its isolation in 1956 from chimpanzee with coryza (8), the significance of RSV as an important human pathogen has been established. Many important mechanisms contributing to RSV infection, replication, and disease pathogenesis have been uncovered; however, there is still no completely safe and effective vaccine or therapeutic treatment against this infection. New and effective disease intervention strategies are urgently needed for controlling RSV infection.

Although the molecular pathogenesis of RSV infection is not completely understood, the host immune responses have been suggested to play an important role in RSV pathogenesis. Robust inflammatory responses are initiated when RSV interacts with respiratory epithelial cells and macrophages through pattern recognition receptors(33, 36, 45) and infects susceptible cells. The inflammatory response is characterized by the upregulation of the expression of inflammatory factors, such as chemokines and cytokines that recruit and activate immune cells (7, 25, 26, 32, 38, 41, 43, 54). The inflammation induced cellular influx adds to the production of inflammatory factors that can help resolve infection but sustained robust inflammatory responses often lead to tissue damage.

Cellular and humoral immunity are two arms of host adaptive responses that are important for controlling and preventing virus infection. Numerous studies in BALB/c mice have shown that cell mediated immunity is important in the resolution of RSV infection, a feature mediated by both CD4 and CD8 T cells. However, natural RSV infection does not lead to sustained protective T cell immunity. There is a rapid loss in the frequency of RSV-specific memory CD8 T cells in the lungs of the infected mice during the resolution phase of infection (9, 11, 47). There is also evidence that CD8+ T cells in the pulmonary infection site in BALB/c mice are functionally inactivated during RSV infection (2, 10, 16), which is consistent with the notion of immune dysregulation associated with RSV infection. Both Th1 and Th2-type responses are induced by RSV infection (3, 51), however a Th2-biased T cell response has been demonstrated in mouse models of infection and in infants (44, 49), a feature that has been suggested to be related to RSV pathogenesis (15, 29, 50, 53).

Given the robust inflammatory response mediated by RSV infection, a reasonable strategy for RSV disease intervention may be to reduce diseases pathogenesis by diminishing the overly robust inflammatory responses while enhancing Th1-type cell responses. Recently, pioneering discoveries have launched RNA interference as a novel, nucleic acid-based therapy against viral pathogens. Specifically, RNA interference (RNAi) is a mechanism that inhibits gene expression by targeting messenger RNA (mRNA) and causing sequence specific degradation or repression of target expression (17, 30, 39, 40). This process is mediated by small interfering RNA (siRNA) that have complementary sequences to the target mRNA. An important step in RNAi function is target site recognition. The antisense strand derived from the siRNA can serves as a guide for a protein complex called RNA-induced silencing complex (RISC) to find the complementary target sequence(28, 37). Recent studies using RNAi therapeutic approaches have

shown that siRNAs targeting the P gene can silence RSV replication (5, 6), thus siRNAs may provide a new disease intervention strategy to reduce the pathology of RSV diseases and improve cellular immunity to RSV.

Although siRNA prophylaxis has been shown to be effective at silencing RSV replication in mice(6), it remained unclear what impact siRNA treatment has on the T cell memory response to RSV re-infection or challenge. Further, it was unclear if siRNA prophylaxis against RSV substantially reduced the pathology of RSV diseases. We hypothesize that RNAi may be an effective disease intervention strategy to silence RSV infection, reduce pathology of diseases, and at the same time attenuate the virus burden but provide sufficient viral antigen allowing for effective vaccination. To test the hypothesis, we determined the dose of siRNA directed against the RSV P gene (siRNA-P) that could be used to reduce but not eliminate RSV replication with a goal being to achieve virus replication similar to levels associated with live attenuated vaccines. We based the approach on observations that 1) siRNAs can be made that effectively target the highly conserved phosphoprotein (P) gene, a key component of the viral RNA-dependent RNA polymerase complex (35), and 2) RNAi silencing is unlikely to completely eliminate all virus, and can be dosed appropriately to reduce but not eliminate all virus. Based on the hypotheses, one experimental focus was to evaluate siRNA-P prophylaxis on the innate and adaptive immune responses to RSV infection with a goal to show that siRNAs can be used a new disease intervention strategy for RSV that allows for effective T cell responses to viral challenge. The specific aims to be addressed:

Specific Aim 1: Determine how siRNA prophylaxis affects RSV viral replication and antigen presentation.

Specific Aim 2: Determine how siRNA prophylaxis impacts RSV mediated lung histopathology following primary and memory responses to RSV challenge.

Specific Aim 3: Determine the frequency, phenotypes, and activation status of immune cells that traffic to the lungs; evaluate T cell responses in the bronchoalveolar lavage (BAL) by measuring cytokine profiles and M2-specific MHC class I tetramer staining after siRNA prophylaxis in both primary and memory responses; determine the quality of the T cell response to RSV challenge.

Another RSV disease intervention strategy is to improve host humoral responses to infection. Since safe and effective RSV vaccines are not currently available, one strategy for disease management has been focused on passive immunotherapy for high-risk patients. Palivizumab, a humanized immunoglobulin G (IgG) monoclonal antibody targeting the RSV F protein is currently used as a passive immune prophylaxis (12, 20). However, passive antibody therapy for RSV is expensive and inconvenient for broad use (42). And this treatment has modest prophylactic efficacy because of the potential genetic variation in seasonal RSV strains, anti-antibody responses and a short half-life of monoclonal antibodies (21, 24, 42), highlighting the need for new disease intervention approaches including the development of safe and effective RSV vaccines.

Several features have been linked to the inability to develop safe and effective RSV vaccines. RSV has been shown to modify aspects of the immune response by various mechanisms (13). The central conserved region of RSV G protein contains a CX3C chemokine motif that has been shown to mimic the activities of fractalkine (52), the only known CX3C chemokine. RSV G protein and fractalkine exists as both membrane-bound and secreted forms (23, 34). Fractalkine (CX3CRL) functions as chemokine in recruiting cells to sites of inflammation as well as providing cell adhesion (23). In particular, it mediates the recruitment

and activation of CX3CR1+ leukocytes including subsets of NK cells, CD4 and CD8 T lymphocytes (31). Infection with RSV G protein mutant viruses lacking a CX3C chemokine motif leads to a substantial increase of pulmonary NK cells, CD4 and CD8 cells compared to wild type RSV (31) suggesting that the CX3C motif of the G protein interacts with CX3CR1 for immune evasion and this manipulation of the immune response may contribute to disease pathogenesis. These findings lead to the hypothesis addressed here that antibodies which block RSV G protein CX3C-CX3CR1 interaction may prevent RSV G protein immune modulation and disease pathogenesis, and lead to new strategies in vaccine development. To address this hypothesis, we evaluated the regions in RSV G protein that induce a protective antibody response which block G protein CX3C-CX3CR1 interaction to provide the foundation for the development of safe and efficacious RSV vaccine candidates. Our working hypothesis is that modifications to the G protein central conserved region which eliminate the CX3C motif may improve vaccine safety while allowing for the induction of antibodies that inhibit infection. Our rationale for these studies is that successful completion would provide the scientific foundation for development of new RSV vaccine strategies to prevent RSV disease. The specific aims to be addressed**:**

Specific Aim 1: To establish an in vitro model to evaluate RSV G protein CX3C-CX3CR1 binding using human 293 cells stably transfected with chemokine receptor, CX3CR1.

Specific Aim 2: To determine which regions in the RSV G protein that induce antibodies against CX3C binding to CX3CR1; and to determine whether antibodies against these epitopes generated in mice immunized with G peptides or polypeptides block the RSV G CX3C binding to CX3CR1.

Specific Aim 3: To determine the ability of antibodies that block G protein CX3C binding to CX3CR1 to inhibit the pulmonary inflammatory responses associated with RSV infection or formalin-inactivated (FI-RSV) vaccine enhanced disease; evaluate the role that blocking antibodies have in ablating enhanced disease or abnormal inflammatory responses to infection in a BALB/c mouse model.

References

1. 2002. Respiratory syncytial virus activity--United States, 2000-01 season. MMWR Morb Mortal Wkly Rep **51:**26-8.

2. **Arimilli, S., E. M. Palmer, and M. A. Alexander-Miller.** 2008. Loss of function in virus-specific lung effector T cells is independent of infection. J Leukoc Biol **83:**564-74.

3. **Becker, Y.** 2006. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy-a review. Virus Genes **33:**235-52.

4. **Billings, J. L., M. I. Hertz, and C. H. Wendt.** 2001. Community respiratory virus infections following lung transplantation. Transpl Infect Dis **3:**138-48.

5. **Bitko, V., and S. Barik.** 2001. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. BMC Microbiol **1:**34.

6. **Bitko, V., A. Musiyenko, O. Shulyayeva, and S. Barik.** 2005. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med **11:**50-5.

7. **Bitko, V., A. Velazquez, L. Yang, Y. C. Yang, and S. Barik.** 1997. Transcriptional induction of multiple cytokines by human respiratory syncytial virus requires activation of NF-kappa B and is inhibited by sodium salicylate and aspirin. Virology **232:**369-78.

8. **Blount, R. E., Jr., J. A. Morris, and R. E. Savage.** 1956. Recovery of cytopathogenic agent from chimpanzees with coryza. Proc Soc Exp Biol Med **92:**544-9.

9. **Chang, J., and T. J. Braciale.** 2002. Respiratory syncytial virus infection suppresses lung CD8+ T-cell effector activity and peripheral CD8+ T-cell memory in the respiratory tract. Nat Med **8:**54-60.

10. **Chang, J., S. Y. Choi, H. T. Jin, Y. C. Sung, and T. J. Braciale.** 2004. Improved effector activity and memory CD8 T cell development by IL-2 expression during experimental respiratory syncytial virus infection. J Immunol **172:**503-8.

11. **Chang, J., A. Srikiatkhachorn, and T. J. Braciale.** 2001. Visualization and characterization of respiratory syncytial virus F-specific CD8(+) T cells during experimental virus infection. J Immunol **167:**4254-60.

12. **Chavez-Bueno, S., A. Mejias, R. A. Merryman, N. Ahmad, H. S. Jafri, and O. Ramilo.** 2007. Intravenous palivizumab and ribavirin combination for respiratory syncytial virus disease in high-risk pediatric patients. Pediatr Infect Dis J **26:**1089-93.

13. **Collins, P. L., and B. S. Graham.** 2008. Viral and host factors in human respiratory syncytial virus pathogenesis. J Virol **82:**2040-55.

14. **Couch, R. B., J. A. Englund, and E. Whimbey.** 1997. Respiratory viral infections in immunocompetent and immunocompromised persons. Am J Med **102:**2-9; discussion 25-6.

15. **Culley, F. J., J. Pollott, and P. J. Openshaw.** 2002. Age at first viral infection determines the pattern of T cell-mediated disease during reinfection in adulthood. J Exp Med **196:**1381-6.

16. **DiNapoli, J. M., B. R. Murphy, P. L. Collins, and A. Bukreyev.** 2008. Impairment of the CD8+ T cell response in lungs following infection with human respiratory syncytial virus is specific to the anatomical site rather than the virus, antigen, or route of infection. Virol J **5:**105.

17. **Doench, J. G., C. P. Petersen, and P. A. Sharp.** 2003. siRNAs can function as miRNAs. Genes Dev **17:**438-42.

18. **Falsey, A. R.** 1998. Respiratory syncytial virus infection in older persons. Vaccine **16:**1775-8.

19. **Falsey, A. R., and E. E. Walsh.** 2000. Respiratory syncytial virus infection in adults. Clin Microbiol Rev **13:**371-84.

20. **Feltes, T. F., and H. M. Sondheimer.** 2007. Palivizumab and the prevention of respiratory syncytial virus illness in pediatric patients with congenital heart disease. Expert Opin Biol Ther **7:**1471-80.

21. **Fenton, C., L. J. Scott, and G. L. Plosker.** 2004. Palivizumab: a review of its use as prophylaxis for serious respiratory syncytial virus infection. Paediatr Drugs **6:**177-97.

22. **Fixler, D. E.** 1996. Respiratory syncytial virus infection in children with congenital heart disease: a review. Pediatr Cardiol **17:**163-8.

23. **Fong, A. M., L. A. Robinson, D. A. Steeber, T. F. Tedder, O. Yoshie, T. Imai, and D. D. Patel.** 1998. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. J Exp Med **188:**1413-9.

24. **Groothuis, J. R., and H. Nishida.** 2002. Prevention of respiratory syncytial virus infections in high-risk infants by monoclonal antibody (palivizumab). Pediatr Int **44:**235-41.

25. **Haeberle, H. A., A. Casola, Z. Gatalica, S. Petronella, H. J. Dieterich, P. B. Ernst, A. R. Brasier, and R. P. Garofalo.** 2004. IkappaB kinase is a critical regulator of chemokine expression and lung inflammation in respiratory syncytial virus infection. J Virol **78:**2232-41.

26. **Haeberle, H. A., R. Takizawa, A. Casola, A. R. Brasier, H. J. Dieterich, N. Van Rooijen, Z. Gatalica, and R. P. Garofalo.** 2002. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. J Infect Dis **186:**1199-206.

27. **Hall, C. B.** 1999. Respiratory syncytial virus: A continuing culprit and conundrum. J Pediatr **135:**2-7.

28. **Hammond, S. M., E. Bernstein, D. Beach, and G. J. Hannon.** 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature **404:**293-6.

29. **Hancock, G. E., D. J. Speelman, K. Heers, E. Bortell, J. Smith, and C. Cosco.** 1996. Generation of atypical pulmonary inflammatory responses in BALB/c mice after immunization with the native attachment (G) glycoprotein of respiratory syncytial virus. J Virol **70:**7783-91.

30. **Hannon, G. J.** 2002. RNA interference. Nature **418:**244-51.

31. **Harcourt, J., R. Alvarez, L. P. Jones, C. Henderson, L. J. Anderson, and R. A. Tripp.** 2006. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. J Immunol **176:**1600-8.

32. **Harrison, A. M., C. A. Bonville, H. F. Rosenberg, and J. B. Domachowske.** 1999. Respiratory syncytical virus-induced chemokine expression in the lower airways: eosinophil recruitment and degranulation. Am J Respir Crit Care Med **159:**1918-24.

33. **Haynes, L. M., D. D. Moore, E. A. Kurt-Jones, R. W. Finberg, L. J. Anderson, and R. A. Tripp.** 2001. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. Journal of Virology **75:**10730-10737.

34. **Hendricks, D. A., K. Baradaran, K. McIntosh, and J. L. Patterson.** 1987. Appearance of a soluble form of the G protein of respiratory syncytial virus in fluids of infected cells. J Gen Virol **68 (Pt 6):**1705-14.

35. **Khattar, S. K., A. S. Yunus, and S. K. Samal.** 2001. Mapping the domains on the phosphoprotein of bovine respiratory syncytial virus required for N-P and P-L interactions using a minigenome system. J Gen Virol **82:**775-9.

36. **Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson, and R. W. Finberg.** 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nature Immunology **1:**398-401.

37. **Martinez, J., A. Patkaniowska, H. Urlaub, R. Luhrmann, and T. Tuschl.** 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110:**563-74.

38. **McNamara, P. S., B. F. Flanagan, L. M. Baldwin, P. Newland, C. A. Hart, and R. L. Smyth.** 2004. Interleukin 9 production in the lungs of infants with severe respiratory syncytial virus bronchiolitis. Lancet **363:**1031-7.

39. **Meister, G., and T. Tuschl.** 2004. Mechanisms of gene silencing by double-stranded RNA. Nature **431:**343-9.

40. **Mello, C. C., and D. Conte, Jr.** 2004. Revealing the world of RNA interference. Nature **431:**338-42.

41. **Miller, A. L., T. L. Bowlin, and N. W. Lukacs.** 2004. Respiratory syncytial virus-induced chemokine production: linking viral replication to chemokine production in vitro and in vivo. J Infect Dis **189:**1419-30.

42. **Moler, F. W., R. W. Brown, R. G. Faix, and J. R. Gilsdorf.** 1999. Comments on palivizumab (Synagis). Pediatrics **103:**495-7.

43. **Noah, T. L., and S. Becker.** 2000. Chemokines in nasal secretions of normal adults experimentally infected with respiratory syncytial virus. Clin Immunol **97:**43-9.

44. **Roman, M., W. J. Calhoun, K. L. Hinton, L. F. Avendano, V. Simon, A. M. Escobar, A. Gaggero, and P. V. Diaz.** 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med **156:**190-5.

45. **Rudd, B. D., E. Burstein, C. S. Duckett, X. Li, and N. W. Lukacs.** 2005. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. J Virol **79:**3350-7.

46. **Simoes, E. A.** 1999. Respiratory syncytial virus infection. Lancet **354:**847-52.

47. **Srikiatkhachorn, A., and T. J. Braciale.** 1997. Virus-specific memory and effector T lymphocytes exhibit different cytokine responses to antigens during experimental murine respiratory syncytial virus infection. J Virol **71:**678-85.

48. **Staat, M. A.** 2002. Respiratory syncytial virus infections in children. Semin Respir Infect **17:**15-20.

49. **Tang, Y. W., K. M. Neuzil, J. E. Fischer, F. W. Robinson, R. A. Parker, and B. S. Graham.** 1997. Determinants and kinetics of cytokine expression patterns in lungs of vaccinated mice challenged with respiratory syncytial virus. Vaccine **15:**597-602.

50. **Tripp, R. A.** 2004. Pathogenesis of respiratory syncytial virus infection. Viral Immunology **17:**165-181.

51. **Tripp, R. A., S. Hou, N. Etchart, A. Prinz, D. Moore, J. Winter, and L. J. Anderson.** 2001. CD4(+) T cell frequencies and Th1/Th2 cytokine patterns expressed in the acute and memory response to respiratory syncytial virus I-E(d)-restricted peptides. Cell Immunol **207:**59-71.

52. **Tripp, R. A., L. P. Jones, L. M. Haynes, H. Zheng, P. M. Murphy, and L. J. Anderson.** 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol **2:**732-8.

53. **Tripp, R. A., C. Oshansky, and R. Alvarez.** 2005. Cytokines and respiratory syncytial virus infection. Proc Am Thorac Soc **2:**147-9.

54. **Zhang, Y., B. A. Luxon, A. Casola, R. P. Garofalo, M. Jamaluddin, and A. R. Brasier.** 2001. Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. J Virol **75:**9044-58.

CHAPTER 2

LITERATURE REVIEW

Respiratory Syncytial Virus

Human respiratory syncytial virus (RSV) is the prototype virus in the *Pneumovirus* genus which classified within the *Pneumovirinae* subfamily of the *Paramyxoviridae* family in the order Mononegavirales. RSV was first identified in 1956 from chimpanzees (18). Since its identification, RSV has become recognized as one of the most important human pathogens causing serious lower respiratory tract disease in infants, young children, the elderly and the immune compromised (2, 15, 40, 60, 62, 64, 86, 189, 194). RSV is the main cause of hospitalization for respiratory tract illness in young children with infection rates about 70% in the first year of life, and 90% can be infected multiple times by 2 years of age (75). RSV also causes severe disease in the elderly with the mortality rate close to influenza virus (59). In the United States, RSV has been estimated to be associated with the hospitalizations of 85,000-144,000 pediatric children and 14,000-60,000 elderly per year (61, 132, 185). Globally, the World Health Organization estimates that RSV leads to 64 million infections and 160,000 deaths annually (1).

RSV is an enveloped single-strand negative-sense RNA virus with a genome of 15.2kb. The genome contains 10 genes encoding 11 different proteins. The structure of RNA genome is schematically shown in Figure 2.1. From the 3’ to 5’ end of the genome, the genes are arranged in the order of two non-structural proteins (NS1, NS2), nucleocapsid (N), phosphoprotein (P), matrix (M), small hydrophobic (SH), surface attachment glycoprotein (G), surface fusion

(a)

(b)

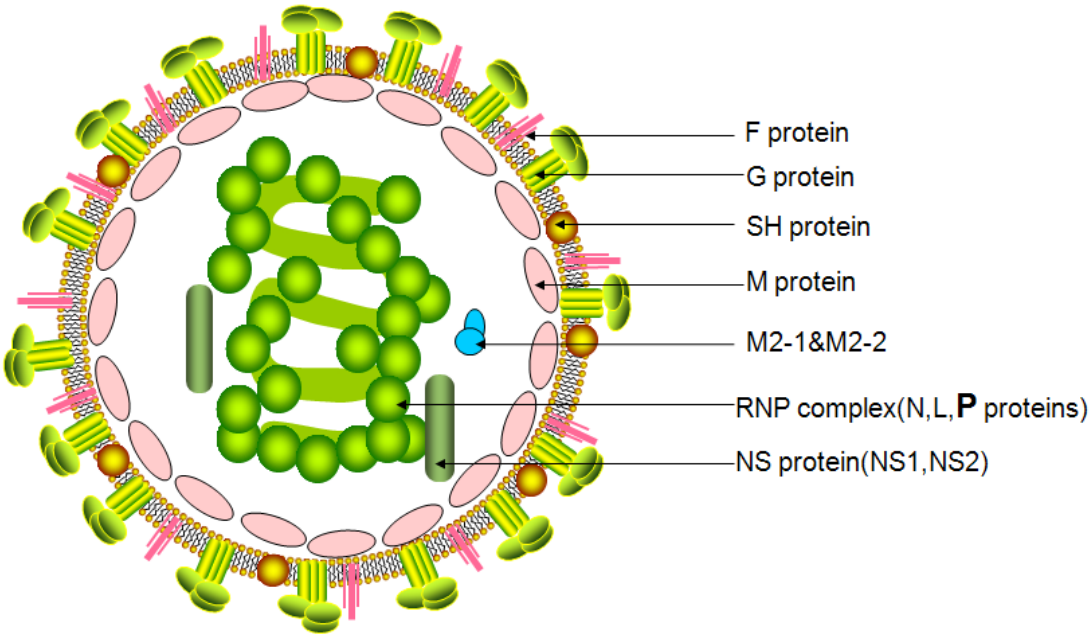


Figure 2.1 Schematic diagram (not to scale) depicting the RSV genome and virion. (a) Schematic of the linear organization of the RSV genome. (b) Schematic of the RSV virion with glycoproteins G and F exposed on the lipid bilayer envelope

glycoprotein (F), a second matrix protein M2 gene and a RNA-dependent RNA polymerase (L). Each gene is transcribed into a separate, capped, polyadenylated mRNA encoding a single viral protein, except the M2 gene contains two overlapping reading frames that are translated into two distinct proteins M2-1/M2-2(79).

RSV virions are pleiomorphic in size and shape (Figure 2.1) and surrounded by a lipid bilayer (8, 104, 154). Inserted in the viral membrane are two major surface membrane glycoproteins, G and F proteins, which are also the major neutralization and protective viral antigens (39). G protein is involved in virus attachment to the cell surface (124), while F protein mediates viral and cell membrane fusion (215). The G glycoprotein is expressed in two different forms: a membrane-bound form (Gm) integrated in the viral membrane, and a secreted form (Gs) secreted by RSV infected cells (93, 95). The Gm protein is a type II glycoprotein containing a single N-terminal hydrophobic domain (amino acids 38-66) that acts as a signal peptide and membrane anchor (126, 168, 214, 218). The C-terminal of Gm ectodomain contains a nonglycosylated central subdomain (amino acids 164-176) and four cysteine residues (residues 173, 176, 182 and 186) which are highly conserved in all RSV isolates. This cysteine region contains a CX3C chemokine motif (amino acids 182-186) which resembles the structure of CX3C chemokine CX3CL1 (fractalkine). The CX3C chemokine motif may aid in virus attachment to the CX3C chemokine receptor (CX3CR1) expressed by some cell types, and/or could mimic CX3C to modify the CX3CL1-mediated immune responses (205). Flanking the central subdomains of the ectodomain are two highly glycosylated variable regions which are extensively modified by N- and O-glycosylation. The soluble form (Gs) lacks the cytoplasmic domain, but retains the same characteristics as Gm such as the CX3C motif, glycosylation and antibody reactivity (94, 95). Figure 2.2 shows the subdomain structures of the RSV G

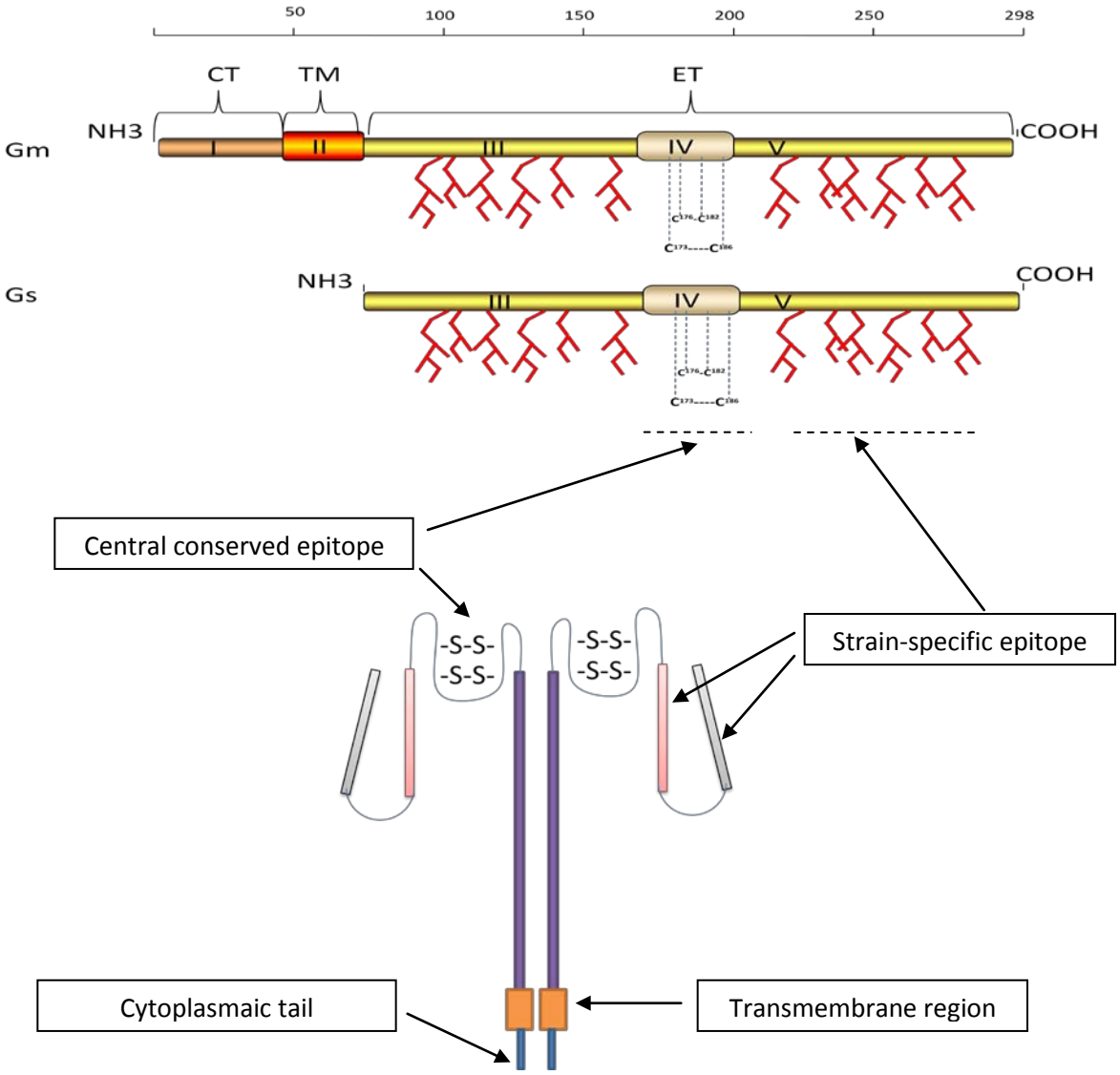


Figure 2.2 Subdomain structures of the RSV G glycoprotein and two-dimentional structure model of membrane G protein (Gm). The 298-amino acid attachment glycoprotein of the A2 strain of RSV is shown subdivided into the following domains: I, a cytoplasmic domain; II, a transmembrane domain; III and V, heavily glycosylated and variable subdomains of the ectodomain; and IV, the nonglycosylated central subdomain of the ectodomain.

glycoprotein and the two-dimensional structure of the Gm molecule. It is illustrated here to be a homodimer, but some studies have proposed that native G proteins could probably be a homotetramer (56). In contrast to G protein as a type II glycoprotein, the RSV F protein is a type I glycoprotein that is synthesized as an inactive precursor (F0). During the transport to the surface of the cell, F0 precursor is cleaved by furin-like proteases into two chains F1 and F2, linked by a disulphide bond (45). The mature F protein is a homotrimer, which functions as fusion protein in the process of viral and cell membrane fusion during infection.

Three viral proteins, N, L and P are the main components of ribonucleoprotein (RNP) complex that are essential for virus replication and transcription. N protein binds tightly to the viral genome forming a nucleocapsid structure (153). L protein is the viral RNA dependent RNA polymerase (RdRp) responsible for both viral genome replication and transcription (195). As a co-factor of viral RNA polymerase, P protein has a critical role for viral RNA synthesis (111) where it is thought to interact with N and L protein to facilitate the RdRp to access and interact with the viral RNA (71). The phosphorylation of P protein is required for efficient RdRp activity by helping stabilize the transcription initiation complex (54). The viral M2 gene encodes two proteins, M2-1 and M2-2, both of which play important roles for virus replication. M2-1 protein acts as a transcription anti-terminator allowing synthesis of full-length mRNA (37). M2-2 protein is associated with translation control and modulation of the switch between viral genome replication and transcription. The SH protein is a small surface protein. It functions as a cation-selective ion channel which resembled the M2 protein of influenza virus (70) and has been shown to interact with the G protein to form a complex on the surface of infected cells (131). The other two RSV proteins, NS1 and NS2, are known to act cooperatively to antagonize the type I (interferon) IFN antiviral response (78, 182, 192, 193).

RSV Replication Cycle

The steps of RSV replication are depicted in Figure 2.3. RSV replication initiates through G protein binding to cell surface components, primarily via heparin-binding domains on the G protein with cell surface glycosaminoglycans (GAG) (20, 63, 116). After attachment of the virion to the cells, F protein is activated by conformational changes to trigger fusion of viral and cell membranes (7). Following cell fusion, the viral nucleocapsids and polymerase are internalized into the cell cytoplasm (6, 97, 123, 170) where the polymerase initiates viral transcription and replication. Although RSV mutant virus studies have shown that the G protein may not be necessary for viral infection and infection can initiate through F protein (107, 201, 203, 207), the G protein appears to be required for efficient viral replication in vivo (203). Genome replication and transcription of the ten viral genes occurs in a 3' to 5' order from a single promoter near the 3' end and proceeds in a sequential and polar manner from the 3'-end of the genome by terminating and reinitiating at each of the gene junctions. This results in a gradient expression of a series of subgenomic mRNAs with the genes at the 3' end of the genome being transcribed more frequently than the genes at the 5' end (38, 118). As the level of protein expressed is related to mRNA abundance, this results in decrease protein production from the genes at the 3' end to the gene at the 5' end (52). Eventually, different viral gene products localize and accumulate at the cell plasma membrane where the progeny virus are assembled and released.

Host Innate Immunity to RSV Infection

Recognition of RSV by the innate immune system

The innate immune system senses pathogen invasion via pattern recognition receptors (PRRs) which recognize conserved pathogen-associated molecular patterns (PAMPs) (138). These

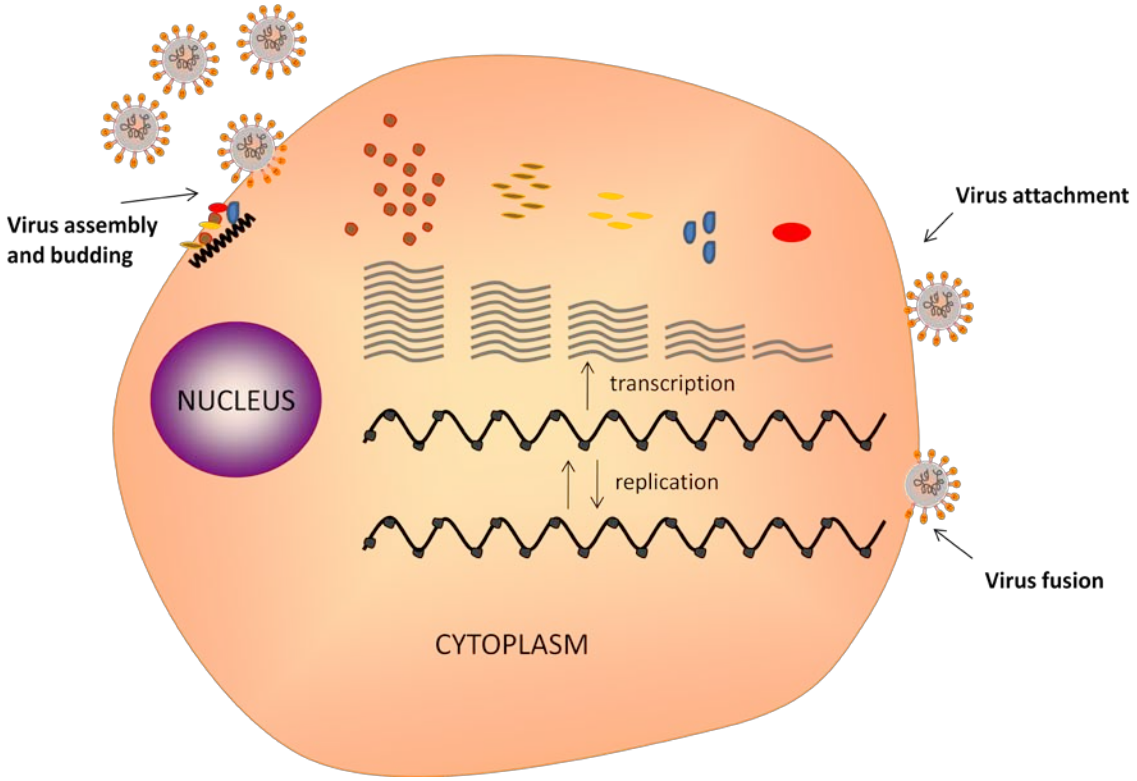


Figure 2.3 Diagram of RSV replication cycle. Virus replication initiates from virus attachment to the cells followed by cell fusion via viral F protein. RNA polymerase initiates virus transcription and genome replication in cells. Different viral gene products are localized and accumulated in the cell plasma membrane where the progeny virus is assembled and released.

PRRs can be the membrane bound such as Toll-like receptors (TLRs), mannose receptors and scavenger receptors or cytoplasmic PRRs like the NOD-like receptors and RIG-I-like RNA helicase (RLH). PRRs are broadly distributed in airway epithelium cells which are the main targets of RSV infection and are distributed on alveolar macrophages and dendritic cells. TLRs are constitutively or inducibly expressed on variety of different cells, including human lung epithelial cells and especially the antigen presenting cells (APCs). TLRs can be expressed on the cell membrane or located intracellularly. Different TLRs appear to be involved in sensing different pathogen types. In contrast to TLRs, RLH survey the cytoplasm for viral RNA. RIG-I and MDA5 are important in the recognition of distinct sets of RNA viruses. Multiple PRRs have been shown to be involved in triggering innate immune responses against RSV infection (69, 84, 120, 128, 144, 151, 183, 186). RSV F protein induces innate immune response by macrophage and DCs through CD14 and TLR4 signaling (119). The importance of TLR4 recognition of RSV is demonstrated in the experiment with TLR4-deficient mice which had the longer viral persistence compared to the normal mice (119). TLR3 is located on the intracellular membrane and detects double stranded RNA (dsRNA). TLR3 expression is up-regulated in target cells following by RSV infection and signals through a MyD88-independent pathway (171). Although TLR3 has not been shown to be critical for RSV clearance, it seems to be important for maintaining an appropriate Th1-type immune environment avoids the Th2-mediated pathology in the lungs of infected mice (172). A recent study has shown that TLR2 is involved in RSV recognition and subsequent activation of the innate immune system (150). In addition, the cytoplasmic PRR, RIG-I but not MDA5, has been shown to be essential in mediating innate immune responses against RSV infection and other paramyxovirus (129). Further, cells lacking RIG-I were shown to be generally more permissive to RSV infection.

Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells (APCs). The co-stimulatory or inhibitory molecules expressed on the surface of DCs and the cytokines they secrete in part determine the destiny of the T cells they activate — whether the T cells are activated or tolerized and whether they are polarized to Th1, Th2 or T regulatory cells (49). Respiratory DCs are located within the intra-eptithelial layer of the respiratory tract and underneath the respiratory epithelium basement membrane in the lamina propria where they meet the pathogen and carry the antigens to the draining lymph node. There are two main subsets of DCs: myeloid or conventional DCs (cDCs, CD11b+, CD11c+) and plasmacytoid DCs (pDCs, CD11b-, B220+). The balance between cDC and pDC in the lung and the draining lymph node has been shown to be crucial for the pulmonary immunity to RSV infection (190). Increased pDC numbers have a protective impact on the nature of the overall immune environment and depletion of pDC from the lungs of RSV infected mice results in a pathologic response skewed towards Th2 cytokine profile (190, 191, 216). Not only the number of DCs but also the quality of antigen presentation by DCs affects the T cell activation. DCs themselves can also be infected by RSV. Although infected DCs can still go differentiation and maturation, RSV-infected DCs can impair T cell activation. Several mechanisms have been suggested including the modulation of soluble mediators, such as IFN-α (164) , or IL-1Rα (177) or other unidentified mediators (47). Additionally the F protein expressed on RSV infected DCs may inhibit T cell activation by direct contact (179). Moreover, a recent study proposed that RSV impairs T cell activation by preventing synapse assembly with DCs (77). This group demonstrated that RSV suppression of T cell activation was not due to mediation of DC-derived soluble factors, but due to the interaction

with RSV-infected DCs, which rendered T cells unresponsive to subsequent TCR engagement and impaired DC-T cell synapse assembly (77).

Macrophages

Macrophages are the key effector cells of the innate immune response. The lower respiratory tract abounds with alveolar macrophages which serve as the frontline of cellular defense against respiratory pathogens. Macrophages are also significant sources of important pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 following RSV infection (12). Depletion of macrophages significantly inhibits the early release of inflammatory cytokines into the airways after RSV infection and enhances the peak viral load in the lung but has little effect on the T cell recruitment and overall lung diseases suggesting that macrophages might only play essential role in the earlier response to RSV infection, but had little effect on the later adaptive response (165). In contrast, another study has shown that the deficiency of alveolar macrophages in NZB mice were central to enhanced disease, because depletion of alveolar macrophages in BALB/c mice before RSV exposure resulted in airway occlusion and a similar pathology was observed in macrophage deficient NZB mice (167).

NK cells

Natural killer (NK) cells constitute a major component of the innate immune system. They have a role in clearance of tumors and virus-infected cells by benefit of their natural cytotoxic ability. Chemokines, such as MIP-1α, are important for the recruitment of NK cells to the site of infection (198). During RSV infection, NK cells are recruited to the lungs very early after infection and reach peak levels at about day 3-4 post-infection (204, 207). DCs are considered to be the primary cell types that potentiate NK cell activation and cytotoxicity (146, 147); however, a recent study showed that alveolar macrophages are required to recruit and

activate NK cells in response to RSV infection, and depletion of macrophages reduced the activation and recruitment of NK cells (165). Interestingly, it has been shown that RSV lacking G and SH proteins exhibits enhanced NK cell infiltraion into the lungs (207). Beside the cytotoxic potential, NK cells produce IFN-γ following antigen recognition that plays an important role in the subsequent adaptive response by enhancing the differentiation of CD8 T cells into effector cytotoxic T lymphocyte (CTL) and CD4 T cells into Th1 cells. Additionally, depletion of asialo-GM+ NK cells leads to a prolonged RSV shedding from infected mice, suggesting an important role of NK cells in RSV viral clearance.

NK T cells

Natural killer T cells (NKT) are a distinct lineage of T cells that express a semi-invariant T cell receptor (TCR) and share a number of cell surface markers with NK cells (25). They recognize glycosphingolipids presented by the non-polymorphic CD1d antigen presenting molecule that is evolutionarily related to the classical major histocompatibility complex (MHC) class I and class II glycoproteins (14, 24). These cells can produce Th1- and Th2-type cytokines and therefore have the potential to impact adaptive immune responses by governing aspects of the cytokine microenvironment. NK T cells have been implicated in immune responses against RSV infection, where NK T cells were shown to have a role in early IFN-γ production and efficient induction of CD8 T cell responses during primary RSV infection (103).

Host Adaptive Humoral Immunity to RSV Infection

RSV infection induces antibody responses against several viral antigens; however, only the two major surface glycoproteins (F and G proteins) induce antibodies which have a major role in protection against RSV infection (83). Vaccination studies using recombinant vaccinia virus (rVV) expressing various RSV proteins have shown that serum antibodies can be induced

by F, G, M2 and P proteins, but only F and G protein were the major determinants of protection(39). The RSV F protein has two forms: a mature form, found in virions, and an incorrectly folded forms that lack important neutralization epitopes (122, 130). Although both of these forms are able to presented to the immune system and induce antibody responses of comparable magnitudes (176), the substantial antibody response against the incorrectly folded form of F protein might lead to diversion or reduction of a protective antibody response against the conformationally correct protein. Comparing F to G protein among RSV isolates, the G protein is the more divergent protein with only 53% identity for G protein and 90% for F protein (35). Therefore, few G-specific monoclonal antibodies are cross-reactive (35), while the majority of F specific monoclonal antibodies are cross-reactive. Unexpectedly, very few individual G protein-specific monoclonal antibodies efficiently neutralize RSV infectivity, and G protein-specific antibody neutralization requires multiple antibodies (139). Further, the majority of G protein-specific monoclonal antibodies are much less effective than F protein-specific monoclonal antibodies in neutralization of RSV. It appears that protective anti-G protein antibodies recognize the central conserved cysteine-rich region of the G protein (209). It is possible that this feature may be linked to antibody-mediated inhibition of G protein CX3C interaction with CX3CR1 and immune modulation (205). Based on their reactivity with specific monoclonal antibodies against the RSV F and G proteins and the different sequences of some genes, RSV is divided into two subgroups: A and B. However, RSV exhibits a single serotype, as there is only three- to four fold difference in reciprocal cross-neutralization in vitro. The two groups, independently, circulate in the human population with predominant subgroup A causing more acute illness (23) .

Neutralizing antibodies have an important role in protection from RSV infection, although serum and mucosal neutralizing antibodies seem to provide different levels of protection. As serum antibodies, mainly composed of IgG, gain access to the lungs much easier to the nasal passages through transduction, they provide better protection of lungs than upper respiratory tract (URT). Passive immunization studies in cotton rats have shown that serum antibody can provide complete protection against RSV replication in the lungs, but only a partial reduction in nasal virus titer (166). Mucosal secretory IgA antibody may have a more important role in local protection, although this antibody is short-lived and have less neutralizing activity comparing to serum IgG antibodies. Repeated RSV infection can induce a sustained antibody response associated with high levels of mucosal IgA in nasal secretions, which can limit virus replication in URT, independent of the level of serum antibodies (141).

Host Adaptive Cellular Immunity to RSV Infection

Although antibody responses are vital for protection against RSV infection, T cell mediated cellular immune responses are of greater importance in virus clearance. In humans, CD8+ T cells recognize F, M, M2 and NS2 proteins, but there is little or no recognition of G, P or NS1 protein. In BALB/c mice, CD8+ CTL primarily recognize F, N and M2 proteins (158). M2 protein contains an immunodominant H-2K^{d} epitope in BALB/c mice (117), and a H-2D^{b} restricted CTL epitope in C57B1/6 mice (174). The identification of CTL epitopes in different models is important for analyzing the kinetics and immunodominance of CTL responses. Priming of different subsets of CD4+ T cells appears to contribute to the quality and magnitude of the CD8+ T cell response and subsequent disease pathogenesis. Studies have demonstrated F and G prime different subset of CD4 T cells in BALB/c mice vaccinated with different recombinant vaccinia virus (rVV) expressing G or F proteins (3). Differentiation and activation

of different subset of CD4 T cells contribute to the Th1/Th2 environment by secreting Th1/ Th2 type of cytokines. In BALB/c mice, F protein primes both CD8+ and CD4+ T cells toward a Th1-type biased cytokine response while G protein primes only CD4+ T cells that are biased towards to Th2-type cytokine response (102). The distribution of CD4 T cell epitopes against the F protein vary in different hosts. The CD4 T cells immunodominant epitope of the G protein is within the regions 162-179 in the non-glycosylated ectodomain. Studies on CD4 T cell epitopes on G protein have been focused on the Th1 and Th2 responses. One study demonstrated that the epitopes in the non-glycosylated ectodomain of G protein are recognized by Th2 type of CD4 T cells in mice, but are poorly recognized by human CD4 T cells (89, 200). Other studies reported that the immunodominant peptide of G protein is recognized by both Th1 and Th2 CD4 T cells in humans (48, 50).

During the primary immune response to RSV infection, the influx of CD4+ and CD8+ T cells is preceded by an early pulmonary NK cell response, however the T cell response peaks between 7-10 days post infection (99). CD8 T lymphocytes play a major role in the clearance of virus. RSV-specific CD8 T cells are found both in the lungs and in the peripheral blood after RSV infection. Studies have shown that virus clearance is temporally associated with the increase of RSV-specific CD8 CTL activity in the lungs (199). Although T cell responses predominantly occur in the lungs during RSV infection, it has been shown in the mouse model that T lymphocyte subsets can redistribute from the peripheral blood to the lung and bronchoalveolar lavage (BAL) after RSV infection (113). Studies also indicate that higher proportion of RSV-specific CD8 T cells in the peripheral blood can be detected in elder infants than younger infants. This might be due to immune immaturity, the Th2 environment in the lung and the suppressive environment associated with maternal antibody in infants.

A T cell memory response is important for RSV-reinfection. The importance of CD4+ memory T cells to RSV-reinfection has been investigated; however, the majority of studies have focused on the response to RSV G protein priming. It has been shown that the memory CD4+ T cells responsive to the RSV G protein in the lungs of primed BALB/c mice challenged with RSV is dominated by effector T cells expressing a single T cell receptor (TCR) Vβ-chain, Vβ14 (213). CD4+ T cells expressing TCR Vbeta14 preferentially proliferate and expand into activated effector T cells in the lungs rather than the lymph nodes which drain the site of infection (220). Although this study is limited to a specific inbred strain of mice, these findings may be important as RSV-specific CD4+ memory T cells have been shown to have a major role in RSV-induced immunopathology, a feature linked to polarizing for a Th2-type cytokine response and pulmonary eosinophilia (26, 30, 80, 82, 204). It has been recently shown that RSV-specific memory CD8 T cells, when present in sufficient numbers, inhibit Th2-associated chemokines, CCL17 and CCL22, and may alter the trafficking of Th2-type cells and eosinophils into the lung (155). Interestingly, the memory CD4+ T cell response to RSV F protein is much broader than that to RSV G protein. Immunization of mice with the F protein elicits a broad repertoire of RSV F-specific CD4+ T cells that predominantly express Th1-type responses; however, in the absence of IFNγ, RSV F-specific memory CD4+ T cells secrete IL-5 and develop pulmonary eosinophilia after RSV challenge suggesting that IFNγ can modulate the memory CD4+ T cell response to secondary RSV infection (29).

CD8+ memory T cells are important for clearing RSV reinfection. Studies of RSV-specific CD8+ memory T cells in human have shown that most pulmonary CD8+ T cells are retained in the lungs and a minority in the peripheral blood (46). Consistent with these findings, it has been shown that 20% of pulmonary CD8 T cells after acute infection are secreting IFNγ in

response to a single immunodominant peptide compared to CD8 T cells in the draining lymph node where only 2-3% secrete IFNγ (159). It remains unclear whether resident or recruited RSV-specific CD8+ T cells may be more important to control RSV reinfection; however, it has been shown that although there is higher proportion of CD8+ memory T cells in the lungs, amplification of recall responses in the organized lymphoid tissue is more efficient (159). This suggests that during RSV infection pulmonary CD8 T cells might be functional impaired compared to the CD8 T cells in the peripheral blood and lymphoid tissue. There is evidence that pulmonary CD8+ T cells from BALB/c mice are functionally inactivated in RSV infection while effector CD8 T cells from lymph nodes or spleen had no defect in function (31). The mechanisms contributing to pulmonary CD8+ T cell functional impairment during RSV infection is not very clear. One study suggested that the functional inactivation of CD8 T cells is associated with T cell receptor (TCR) signaling and the activity could be improved by IL-2 expression in the lungs (32). Another study suggested that the functional inactivation of CD8 T cells is independent of RSV infection and is mediated by the immunosuppressive agents in basal lung environment that promote the lost of function of CD8 T cells in the lungs (5). Moreover, a recent study suggested that healthy lung epithelial cells independent of RSV infection contribute to the inhibition of T cell activation (217).

Cytokines response during RSV infection

Cytokines are a diverse group of low molecular weight, soluble secreted proteins that are produced in response to immune stimuli and function as chemical messengers for mediating and regulating innate immunity, adaptive immunity and as well as hematopoiesis. Chemokines are group of cytokines that enable the migration of leukocytes from the blood to the tissues at the site of inflammation. During RSV infection, a wide range of cytokines and chemokines are

produced by different cell types. For example, RSV infection of the cells results in the activation of NF-κB activation, which leads to the production of various inflammatory chemokines and cytokines including RANTES(CCL5), MIP-1α(CCL3), MCP-1(CCL2), eotaxin(CCL11), IL-8(CXCL8), TNF-α, IL6 etc., which contribute the inflammation by recruiting neutrophiles, macrophages and lymphocytes to the lung airways (140). The chemokines and cytokines can either be induced by virus infection directly or via the autocrine/paracrine feedback effects from other cytokines. For example, fractalkine, the only known CX3C chemokine, is secreted by human endothelial cells activated by proinflammatory signals (TNF-α, IL-1, lipopolysaccharide, CD40 ligand, IFN-γ) (11, 66).

Certain patterns of cytokine and chemokine expression in a RSV-infected individual have been suggested to be an indicator of disease severity (98). Studies have shown RSV-infected patients present increased levels of MIP-1α, RANTES and IL-8 in the upper and lower respiratory tract (91). Blocking any one of these factors may result in less severe disease, which has been demonstrated in the mice infected with RSV that antibody-mediated depletion of RANTES or eotaxin have resulted in reduced airway hyperreactivity (AHR) and eosinophilia in (137, 202). Severe disease manifestations during RSV infection have been associated with a Th2-type cytokine response (44). It has been shown young children are prone to develop Th2 bias cytokine response, which has also been associated with RSV pathology (169). However, there are other studies demonstrating a predominant Th1 type (19) or Th1/Th2 mixed response (206).

Virus-host cell interaction and viral evasion

RSV infects a number of different cell lines *in vitro* while it primarily infects respiratory epithelial cells lining the nasal passages and respiratory tract *in vivo*. Multiple aspects of host

cells are affected by RSV infection to facilitate virus replication as well as by host anti-viral response to provide defense against virus attack. RSV infection has been shown to alter cytokine and chemokine expression as well as the tempo and expression patterns of various cytokine genes that affect protein metabolism, cell growth and proliferation, cytoskeleton organization and regulation of nucleotides and nucleic acid synthesis (136, 224).

To overcome the host immune defenses, RSV has enlisted a variety of immune modulatory and evasion strategies to promote virus replication. By delaying programmed cell death, RSV facilitates its replication in cells, a feature that has been linked to the ability of RSV to induce expression of anti-apoptosis genes including the anti-apoptotic gene IEX-1L, the B-cell leukemia/lymphoma 2 (Bcl-2) family genes Mcl-1 and Bcl-XL (53, 115, 127, 143). Other mechanisms have also been suggested to be associated to the inhibition of tumor suppressor p53 and Akt activation (85). RSV nonstructural proteins (NS1 and NS2) and SH protein are considered to be main viral factors in viral anti-apoptosis effect (17, 67). RSV can also facilitate its replication in host cells through the modulation of the structure and function of respiratory epithelial cells. RSV infections can enhance the expression of MMP-9, which is involved in the digestion of extracellular matrix resulting in increased rate of RSV syncytium formation which facilitates viral replication (222). RSV infection also results in reduced levels of surfactant proteins (SP), which are pattern recognition molecules produced by alveolar and airway epithelial cells to form the first line defense against virus infection in lung by facilitating the opsonization and receptor mediated uptake of RSV (110, 211).

RSV-mediated modification of the cytokine response has been extensively studied. It is well-established that IFN-α/β are produced by most cells during the early response to virus infection. These type I IFNs are secreted by infected cells and bind to the neighboring cells

resulting in the development of an anti-virus state. RSV has been shown to be a poor inducer of IFN-α/β and resistant to the anti-viral effect of IFN-α/β (182). Many of these features are linked to RSV NS1 and NS2 proteins which act cooperatively to prevent the induction of IFN-α/β by blocking the activation of IFN regulatory factor 3 (IRF-3) and by inhibiting type I IFN-induced (STAT)-2 signaling pathway (36, 182). Cells infected with RSV lacking NS1 and NS2 genes have been shown to express higher levels of IFN-α/β and are more immunogenic (210). As IFN α/β plays an important role in DC maturation, activation of NK cells, differentiation and function of T cells, and enhance primary antibody responses, the inhibition of IFN production by RSV has substantial negative impact on the subsequent adaptive immune response. Despite the dominant role of NS1/2 genes in IFN antagonism, it has been shown that the RSV G protein also inhibits IFNβ through a mechanism linked to the induction of suppressor of cytokine signaling proteins (SOCS), which negatively regulate cytokine expression (41, 223).

The G protein has several known attributes that contribute to immune evasion. The two subdomains of the G protein ectodomain flanking the central conserved region are highly glycosylated; however the pattern of glycosylation changes depending on the specific cell type infected (72, 73, 160, 161). As the antibody response primarily recognizes epitopes within the C-terminal region of the G protein (135, 173), the altered glycosylation patterns likely contribute to immune evasion associated with changes of the G protein antigenic profile (27, 28). Host protein mimicry as an immune evasion mechanism is also associated with RSV G protein. For example, the Gm and Gs proteins both contain a central conserved cysteine-rich region (GCRR) that has homology to the fourth subdomain of the tumor necrosis factor (TNF) receptor. TNFα/β are proinflammatory cytokines involved in a large range of inflammatory conditions (21) and in the antiviral response to RSV infection (157). It is possible that RSV G protein may bind to TNFα or

other homologues modulating the host antiviral response (95, 121). For example, RSV G protein can inhibit TLR4-mediated cytokine production by inhibiting nuclear translocation of NF-κB, which has been suggested to be related to the conserved cystein-rich region that mimics the fourth domain of the TNF receptor (121). The GCRR also contains a CX3C chemokine motif at amino acid positions 182-186, which resembles the fractalkine and competitively binds to CX3CR1 receptors (90). By CX3CR1 mimicry RSV G protein act as a antagonist of fractalkine to facilitate virus replication by altering CX3CL1 chemotaxis of human and mouse leukocytes (205). It has been shown that the expression of G protein during RSV infection of mice is related to the decreased number of activated and RSV-specific pulmonary $CX3CR1^{+}$ T cells, as well as NK cells (90). Consistent with this finding, studies in mice infected with a RSV mutant virus lacking the G and SH genes demonstrated enhanced numbers of NK cells recruited to the lung as well as increased IFNγ and TNFα production (207). Together these studies suggest that RSV G protein can modulate both the innate and adaptive immune responses.

RSV Disease Pathogenesis

RSV disease pathogenesis mechanisms are not well understood. The relative contribution of viral versus various host factors to RSV pathogenesis is still controversial. Some of the known risk host factors for severe disease include the age of infection, genetic predisposition, pathogenic features of host immune responses (51, 57, 58). Some of the viral factors include high infectivity, tissue tropism, and the multiple viral evasion strategies.

RSV infects infants very early in life, which increases the impact of RSV. Very young infants are less tolerant of severe infection than older individuals. One of the important reasons is because of the lower magnitude and poor durability of RSV antibody response. The reduced immune responses are most probably due to the combination of immune suppression by RSV-

specific maternal serum antibodies (43) and immunologic immaturity (152). Genetic predisposition has been indicated in the studies associating genetic polymorphisms in a number of genes encoding cytokines, chemokines and proteins involved in surface interactions or intracellular signaling, with susceptibility to RSV disease (34, 65, 219).

There are numerous lines of evidence which indicate that the early inmate host response to primary infection is important for RSV pathogenesis. Overly robust inflammatory responses are strongly implicated in RSV pathogenesis, which is documented by clinical studies that increased expression of inflammatory mediators or their mRNAs in respiratory secretions in RSV patients compared to normal persons. The over-exuberant or inappropriate elaboration of immune mediators in the respiratory tract may exacerbate the inflammatory response and promote airway damage and pathogenesis during virus clearance. Further, neutrophils involved in the Th2-mediated or inflammatory responses are suspected to have a role in RSV pathogenesis based on the observation that increased number of neutrophils in lung tissues from patients with enhanced RSV diseases subsequent to vaccination of formalin-inactivated RSV (FI-RSV). Excessive T-lymphocyte cytotoxicity has also been suggested to be one potential mechanism of immune-mediated pathogenesis. T cell response help resolve RSV infection, but also contribute the disease pathgoenesis, as has been indicated in small animal models in which ablation of either CD4+ or CD8+ T cells after RSV infection has resulted in long-term virus infection with decreased disease severity and illness (55, 81). There is suggestive but inconsistent evidence of a role for Th2-biased responses in RSV pathogenesis. There is considerable evidence of a Th2-type biased immune response specific for some RSV antigens (13, 55, 82, 134, 156, 212). However, other groups have reported a Th1-biased response or mixed responses associated with severe pediatric RSV disease (22, 74, 142).

The virus factors may represent the main factors of RSV pathogenesis. RSV is one of the most contagious human pathogens with infectivity rate comparable to measles virus. RSV is readily introduced and spreads with ease, which is demonstrated in studies that the natural introduction of RSV into a day-care setting resulted in the infection of more than 90% of infants and children (105). Despite limited antigenic variation, RSV is still able to reinfect throughout life and even in the same epidemic season (87). RSV has been shown to modify the tempo and magnitude of cytokine and chemokine expression patterns during infection (13, 55, 82, 145, 204, 208) via multiple immune evasion strategies, which contribute to the aspects of disease pathogenesis described above.

There is no single paradigm for explaining RSV disease pathogenesis, which is a multi-factorial process involving not only host but viral factors such as virus replication, innate responses to infection, and aberrant immune responses linked to modification by RSV proteins. A better understanding of the interplay between RSV and the host response to infection is needed to facilitate the development of disease intervention strategies against RSV infection.

Development of RSV vaccine

Since the discovery of RSV, the development of an RSV vaccine has been recognized as a priority (114, 163). However, despite over 40 years of effort, there is still no effective and safe vaccine available (114, 163). A successful RSV vaccine requires it to provide effective and protective immune responses while ensuring vaccine safety. The first candidate vaccine, FI-RSV, lead to more severe disease in vaccinated children following subsequent natural infection (33, 68, 106, 112). The experience with the FI-RSV vaccine has generated a great deal of concern about the safety of any non-live RSV vaccine and led to an extremely cautious approach for RSV vaccine development. The search for new vaccine candidates such as live-attenuated vaccines

has been invigorated with advent of reverse genetics. Unfortunately, none of the live-attenuated vaccine candidates have been shown to be both safe and effective (108, 109, 221). The disadvantages of live-attenuated vaccine such as poor growth and physical instability make the manufacture of this type of vaccine less feasible. Subunit vaccines have been the focus of vaccine development for RSV-experienced individuals such as older children and adult populations (163). However, subunit vaccines to date have provided incomplete protection in the upper respiratory tract. RSV DNA vaccines have also been evaluated, but they have not been very effective in the most trials.

There are several obstacles that impede RSV vaccine development. Natural infection does not confer full protection from re-infection and provides only partial protection from disease, suggesting it will be difficult to induce a protective immune response (76, 88, 92). The immaturity of immune system in the young infants makes it difficult to induce a protective immune response. The presence of maternal antibody in infants partly suppresses the immune responses to infection. The mechanism is not known, but it primarily affects humoral instead of cell-mediated immunity (42, 187, 188). Antigenic and genetic differences among circulating RSV strains(4, 149, 162) are likely sufficient to affect the level of cross protection induced by viruses from different groups, and it is not known if subgroup differences affect the level of cross protection induced by viruses from different subgroups within the same group (96, 101, 148).

RNA interference and disease intervention

The process of RNA interference (RNAi) is evolutionarily conserved, gene-silencing mechanism in which small 19-23 nucleotide double-stranded RNA molecules, or small interfering RNA (siRNA), targets cognate RNA for destruction with exquisite potency and selectivity causing post-transcriptional gene silencing (9, 125, 184). The RNAi machinery is

expressed in all eukaryotic cells and has been shown to regulate the expression of key genes involved in cell differentiation in plants and animals (133, 175, 178, 181). Given the power of RNAi to silence genes, numerous RNAi drugs are being developed as an effective disease intervention strategy to inhibit viral replication, particularly for RNA viruses such as HIV and respiratory syncytial virus (16, 184, 196). Development of synthetic siRNA drugs is particularly useful in situations in which long-term silencing is not required or undesirable, e.g. treating acute viral infections. RNAi has been shown to work effectively as an anti-viral agent (125, 178, 184, 197), and this breakthrough technology emerges as a powerful tool to protect humans from viral infection.

RNAi therapeutics offers numerous advantages over conventional anti-viral drugs. For example, it is generally easier and more flexible to develop siRNAs to target cognate mRNA because the target mRNA and siRNA are sequence-specific, complementary, and for the target mRNA, siRNA inhibition can be achieved by targeting different regions of the mRNA. Given the high homology of siRNA to the target region there is selective destruction of only interested transcript without adverse side effects and minimal if any detectable off-target effects. Also, siRNAs without suitable targets remain inert within cells, and for effective gene silencing, only substoichiometric amounts of siRNA are needed to target mRNA. In addition, siRNAs targeting conserved cognate mRNA are similarly effective in different species (125, 178, 184, 197). Importantly, several siRNA delivery systems including aerosol, intravenous, and topical, can be used to distribute siRNAs efficiently into cells of nearly all organs. These qualities have been shown in several studies of siRNA inhibition of viral infection, particularly for paramyxoviruses such as RSV (10, 16, 100, 125, 180, 184, 197).

References

1. 2009, posting date. Acute Respiratory Infections (Update February 2009). World Health Organization. [Online.]

2. 2002. Respiratory syncytial virus activity--United States, 2000-01 season. MMWR Morb Mortal Wkly Rep **51:**26-8.

3. **Alwan, W. H., and P. J. Openshaw.** 1993. Distinct patterns of T- and B-cell immunity to respiratory syncytial virus induced by individual viral proteins. Vaccine **11:**431-7.

4. **Anderson, L. J., J. C. Hierholzer, C. Tsou, R. M. Hendry, B. F. Fernie, Y. Stone, and K. McIntosh.** 1985. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis **151:**626-33.

5. **Arimilli, S., E. M. Palmer, and M. A. Alexander-Miller.** 2008. Loss of function in virus-specific lung effector T cells is independent of infection. J Leukoc Biol **83:**564-74.

6. **Arslanagic, E., M. Matsumoto, K. Suzuki, K. Nerome, H. Tsutsumi, and T. Hung.** 1996. Maturation of respiratory syncytial virus within HEp-2 cell cytoplasm. Acta Virol **40:**209-14.

7. **Bachi, T.** 1988. Direct observation of the budding and fusion of an enveloped virus by video microscopy of viable cells. J Cell Biol **107:**1689-95.

8. **Bachi, T., and C. Howe.** 1973. Morphogenesis and ultrastructure of respiratory syncytial virus. J Virol **12:**1173-80.

9. **Bagasra, O., and K. R. Prilliman.** 2004. RNA interference: the molecular immune system. J Mol Histol **35:**545-53.

10. **Barik, S.** 2004. Development of gene-specific double-stranded RNA drugs. Ann Med **36:**540-51.

11. **Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik, and T. J. Schall.** 1997. A new class of membrane-bound chemokine with a CX3C motif. Nature **385:**640-4.

12. **Becker, S., J. Quay, and J. Soukup.** 1991. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. J Immunol **147:**4307-12.

13. **Becker, Y.** 2006. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy--a review. Virus Genes **33:**235-52.

14. **Bendelac, A., M. N. Rivera, S. H. Park, and J. H. Roark.** 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol **15:**535-62.

15. **Billings, J. L., M. I. Hertz, and C. H. Wendt.** 2001. Community respiratory virus infections following lung transplantation. Transpl Infect Dis **3:**138-48.

16. **Bitko, V., A. Musiyenko, O. Shulyayeva, and S. Barik.** 2005. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med **11:**50-5.

17. **Bitko, V., O. Shulyayeva, B. Mazumder, A. Musiyenko, M. Ramaswamy, D. C. Look, and S. Barik.** 2007. Nonstructural proteins of respiratory syncytial virus suppress premature apoptosis by an NF-kappaB-dependent, interferon-independent mechanism and facilitate virus growth. J Virol **81:**1786-95.

18. **Blount, R. E., Jr., J. A. Morris, and R. E. Savage.** 1956. Recovery of cytopathogenic agent from chimpanzees with coryza. Proc Soc Exp Biol Med **92:**544-9.

19. **Bont, L., C. J. Heijnen, A. Kavelaars, W. M. van Aalderen, F. Brus, J. T. Draaisma, S. M. Geelen, H. J. van Vught, and J. L. Kimpen.** 1999. Peripheral blood cytokine responses and disease severity in respiratory syncytial virus bronchiolitis. Eur Respir J **14:**144-9.

20. **Bourgeois, C., J. B. Bour, K. Lidholt, C. Gauthray, and P. Pothier.** 1998. Heparin-Like Structures on Respiratory Syncytial Virus Are Involved in Its Infectivity In Vitro. J. Virol. **72:**7221-7227.

21. **Bradley, J. R.** 2008. TNF-mediated inflammatory disease. Journal of Pathology **214:**149-160.

22. **Brandenburg, A. H., A. Kleinjan, B. van Het Land, H. A. Moll, H. H. Timmerman, R. L. de Swart, H. J. Neijens, W. Fokkens, and A. D. Osterhaus.** 2000. Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. J Med Virol **62:**267-77.

23. **Brandenburg, A. H., R. van Beek, H. A. Moll, A. D. Osterhaus, and E. C. Claas.** 2000. G protein variation in respiratory syncytial virus group A does not correlate with clinical severity. J Clin Microbiol **38:**3849-52.

24. **Brigl, M., L. Bry, S. C. Kent, J. E. Gumperz, and M. B. Brenner.** 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. Nat Immunol **4:**1230-7.

25. **Brutkiewicz, R. R., Y. Lin, S. Cho, Y. K. Hwang, V. Sriram, and T. J. Roberts.** 2003. CD1d-mediated antigen presentation to natural killer T (NKT) cells. Crit Rev Immunol **23:**403-19.

26. **Bueno, S. M., P. A. Gonzalez, R. Pacheco, E. D. Leiva, K. M. Cautivo, H. E. Tobar, J. E. Mora, C. E. Prado, J. P. Zuniga, J. Jimenez, C. A. Riedel, and A. M. Kalergis.** 2008. Host immunity during RSV pathogenesis. Int Immunopharmacol **8:**1320-9.

27. **Cane, P. A.** 1997. Analysis of linear epitopes recognised by the primary human antibody response to a variable region of the attachment (G) protein of respiratory syncytial virus. Journal of Medical Virology **51:**297-304.

28. **Cane, P. A.** 2001. Molecular epidemiology of respiratory syncytial virus. Reviews in Medical Virology **11:**103-116.

29. **Castilow, E. M., M. R. Olson, D. K. Meyerholz, and S. M. Varga.** 2008. Differential role of gamma interferon in inhibiting pulmonary eosinophilia and exacerbating systemic disease in fusion protein-immunized mice undergoing challenge infection with respiratory syncytial virus. J Virol **82:**2196-207.

30. **Castilow, E. M., M. R. Olson, and S. M. Varga.** 2007. Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. Immunol Res **39:**225-39.

31. **Chang, J., and T. J. Braciale.** 2002. Respiratory syncytial virus infection suppresses lung CD8+ T-cell effector activity and peripheral CD8+ T-cell memory in the respiratory tract. Nat Med **8:**54-60.

32. **Chang, J., S. Y. Choi, H. T. Jin, Y. C. Sung, and T. J. Braciale.** 2004. Improved effector activity and memory CD8 T cell development by IL-2 expression during experimental respiratory syncytial virus infection. J Immunol **172:**503-8.

33. **Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette.** 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am J Epidemiol **89:**449-63.

34. **Choi, E. H., H. J. Lee, T. Yoo, and S. J. Chanock.** 2002. A common haplotype of interleukin-4 gene IL4 is associated with severe respiratory syncytial virus disease in Korean children. J Infect Dis **186:**1207-11.

35. **Collins, P., RM Chanock,BR Murphy (ed.).** 2001. *Fields Virology*. Lippincott-Raven, Philadelphia.

36. **Collins, P. L., and B. S. Graham.** 2008. Viral and host factors in human respiratory syncytial virus pathogenesis. J Virol **82:**2040-55.

37. **Collins, P. L., M. G. Hill, J. Cristina, and H. Grosfeld.** 1996. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. Proc Natl Acad Sci U S A **93:**81-5.

38. **Collins, P. L., and G. W. Wertz.** 1983. cDNA cloning and transcriptional mapping of nine polyadenylylated RNAs encoded by the genome of human respiratory syncytial virus. Proc Natl Acad Sci U S A **80:**3208-12.

39. **Connors, M., P. L. Collins, C. Y. Firestone, and B. R. Murphy.** 1991. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J Virol **65:**1634-7.

40. **Couch, R. B., J. A. Englund, and E. Whimbey.** 1997. Respiratory viral infections in immunocompetent and immunocompromised persons. Am J Med **102:**2-9; discussion 25-6.

41. **Croker, B. A., H. Kiu, and S. E. Nicholson.** 2008. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol.

42. **Crowe, J. E., Jr., C. Y. Firestone, and B. R. Murphy.** 2001. Passively acquired antibodies suppress humoral but not cell-mediated immunity in mice immunized with live attenuated respiratory syncytial virus vaccines. J Immunol **167:**3910-8.

43. **Crowe, J. E., Jr., and J. V. Williams.** 2003. Immunology of viral respiratory tract infection in infancy. Paediatr Respir Rev **4:**112-9.

44. **Culley, F. J., J. Pollott, and P. J. Openshaw.** 2002. Age at first viral infection determines the pattern of T cell-mediated disease during reinfection in adulthood. J Exp Med **196:**1381-6.

45. **Day, N. D., P. J. Branigan, C. Liu, L. L. Gutshall, J. Luo, J. A. Melero, R. T. Sarisky, and A. M. Del Vecchio.** 2006. Contribution of cysteine residues in the extracellular domain of the F protein of human respiratory syncytial virus to its function. Virol J **3:**34.

46. **de Bree, G. J., E. M. van Leeuwen, T. A. Out, H. M. Jansen, R. E. Jonkers, and R. A. van Lier.** 2005. Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung. J Exp Med **202:**1433-42.

47. **de Graaff, P. M., E. C. de Jong, T. M. van Capel, M. E. van Dijk, P. J. Roholl, J. Boes, W. Luytjes, J. L. Kimpen, and G. M. van Bleek.** 2005. Respiratory syncytial virus infection of monocyte-derived dendritic cells decreases their capacity to activate CD4 T cells. J Immunol **175:**5904-11.

48. **de Graaff, P. M., J. Heidema, M. C. Poelen, M. E. van Dijk, M. V. Lukens, S. P. van Gestel, J. Reinders, E. Rozemuller, M. Tilanus, P. Hoogerhout, C. A. van Els, R. G. van der Most, J. L. Kimpen, and G. M. van Bleek.** 2004. HLA-DP4 presents an immunodominant peptide from the RSV G protein to CD4 T cells. Virology **326:**220-30.

49. **de Jong, E. C., H. H. Smits, and M. L. Kapsenberg.** 2005. Dendritic cell-mediated T cell polarization. Springer Semin Immunopathol **26:**289-307.

50. **de Waal, L., S. Yuksel, A. H. Brandenburg, J. P. Langedijk, K. Sintnicolaas, G. M. Verjans, A. D. Osterhaus, and R. L. de Swart.** 2004. Identification of a common HLA-DP4-restricted T-cell epitope in the conserved region of the respiratory syncytial virus G protein. J Virol **78:**1775-81.

51. **DeVincenzo, J. P.** 2005. Factors predicting childhood respiratory syncytial virus severity: what they indicate about pathogenesis. Pediatr Infect Dis J **24:**S177-83, discussion S182.

52. **Dickens, L. E., P. L. Collins, and G. W. Wertz.** 1984. Transcriptional mapping of human respiratory syncytial virus. J Virol **52:**364-9.

53. **Domachowske, J. B., C. A. Bonville, A. J. Mortelliti, C. B. Colella, U. Kim, and H. F. Rosenberg.** 2000. Respiratory syncytial virus infection induces expression of the anti-apoptosis gene IEX-1L in human respiratory epithelial cells. J Infect Dis **181:**824-30.

54. **Dupuy, L. C., S. Dobson, V. Bitko, and S. Barik.** 1999. Casein kinase 2-mediated phosphorylation of respiratory syncytial virus phosphoprotein P is essential for the transcription elongation activity of the viral polymerase; phosphorylation by casein kinase 1 occurs mainly at Ser(215) and is without effect. J Virol **73:**8384-92.

55. **Durbin, J. E., and R. K. Durbin.** 2004. Respiratory syncytial virus-induced immunoprotection and immunopathology. Viral Immunol **17:**370-80.

56. **Escribano-Romero, E., J. Rawling, B. Garcia-Barreno, and J. A. Melero.** 2004. The soluble form of human respiratory syncytial virus attachment protein differs from the membrane-bound form in its oligomeric state but is still capable of binding to cell surface proteoglycans. J Virol **78:**3524-32.

57. **Everard, M. L.** 2006. The role of the respiratory syncytial virus in airway syndromes in childhood. Curr Allergy Asthma Rep **6:**97-102.

58. **Falsey, A. R.** 2007. Respiratory syncytial virus infection in adults. Semin Respir Crit Care Med **28:**171-81.

59. **Falsey, A. R.** 2005. Respiratory syncytial virus infection in elderly and high-risk adults. Exp Lung Res **31 Suppl 1:**77.

60. **Falsey, A. R.** 1998. Respiratory syncytial virus infection in older persons. Vaccine **16:**1775-8.

61. **Falsey, A. R., and E. E. Walsh.** 1998. Relationship of serum antibody to risk of respiratory syncytial virus infection in elderly adults. J Infect Dis **177:**463-6.

62. **Falsey, A. R., and E. E. Walsh.** 2000. Respiratory syncytial virus infection in adults. Clin Microbiol Rev **13:**371-84.

63. **Feldman, S. A., R. M. Hendry, and J. A. Beeler.** 1999. Identification of a linear heparin binding domain for human respiratory syncytial virus attachment glycoprotein G. J Virol **73:**6610-7.

64. **Fixler, D. E.** 1996. Respiratory syncytial virus infection in children with congenital heart disease: a review. Pediatr Cardiol **17:**163-8.

65. **Forton, J. T., K. Rowlands, N. Hanchard, M. Herbert, D. P. Kwiatkowski, and J. Hull.** 2009. Genetic association study for RSV Bronchiolitis in infancy at the 5q31 cytokine cluster. Thorax.

66. **Fraticelli, P., M. Sironi, G. Bianchi, D. D'Ambrosio, C. Albanesi, A. Stoppacciaro, M. Chieppa, P. Allavena, L. Ruco, G. Girolomoni, F. Sinigaglia, A. Vecchi, and A. Mantovani.** 2001. Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. J Clin Invest **107:**1173-81.

67. **Fuentes, S., K. C. Tran, P. Luthra, M. N. Teng, and B. He.** 2007. Function of the respiratory syncytial virus small hydrophobic protein. J Virol **81:**8361-6.

68. **Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn.** 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am J Epidemiol **89:**435-48.

69. **Gagro, A., M. Tominac, V. Krsulovic-Hresic, A. Bace, M. Matic, V. Drazenovic, G. Mlinaric-Galinovic, E. Kosor, K. Gotovac, I. Bolanca, S. Batinica, and S. Rabatic.** 2004. Increased Toll-like receptor 4 expression in infants with respiratory syncytial virus bronchiolitis. Clin Exp Immunol **135:**267-272.

70. **Gan, S. W., L. Ng, X. Lin, X. Gong, and J. Torres.** 2008. Structure and ion channel activity of the human respiratory syncytial virus (hRSV) small hydrophobic protein transmembrane domain. Protein Sci **17:**813-20.

71. **Garcia-Beato, R., I. Martinez, C. Franci, F. X. Real, B. Garcia-Barreno, and J. A. Melero.** 1996. Host cell effect upon glycosylation and antigenicity of human respiratory syncytial virus G glycoprotein. Virology **221:**301-9.

72. **Garcia-Beato, R., I. Martinez, C. Franci, F. X. Real, B. GarciaBarreno, and J. A. Melero.** 1996. Host cell effect upon glycosylation and antigenicity of human respiratory syncytial virus G glycoprotein. Virology **221:**301-309.

73. **Garcia-Beato, R., and J. A. Melero.** 2000. The C-terminal third of human respiratory syncytial virus attachment (G) protein is partially resistant to protease digestion and is glycosylated in a cell-type-specific manner. Journal of General Virology **81:**919-927.

74. **Garofalo, R. P., J. Patti, K. A. Hintz, V. Hill, P. L. Ogra, and R. C. Welliver.** 2001. Macrophage inflammatory protein-1alpha (not T helper type 2 cytokines) is associated with severe forms of respiratory syncytial virus bronchiolitis. J Infect Dis **184:**393-9.

75. **Glezen, P., and F. W. Denny.** 1973. Epidemiology of acute lower respiratory disease in children. N Engl J Med **288:**498-505.

76. **Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel.** 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child **140:**543-6.

77. **Gonzalez, P. A., C. E. Prado, E. D. Leiva, L. J. Carreno, S. M. Bueno, C. A. Riedel, and A. M. Kalergis.** 2008. Respiratory syncytial virus impairs T cell activation by preventing synapse assembly with dendritic cells. Proc Natl Acad Sci U S A **105:**14999-5004.

78. **Gotoh, B., T. Komatsu, K. Takeuchi, and J. Yokoo.** 2001. Paramyxovirus accessory proteins as interferon antagonists. Microbiol Immunol **45:**787-800.

79. **Gould, P. S., and A. J. Easton.** 2007. Coupled translation of the second open reading frame of M2 mRNA is sequence dependent and differs significantly within the subfamily Pneumovirinae. J Virol **81:**8488-96.

80. **Graham, B. S.** 1995. Pathogenesis of respiratory syncytial virus vaccine-augmented pathology. Am J Respir Crit Care Med **152:**S63-6.

81. **Graham, B. S., L. A. Bunton, P. F. Wright, and D. T. Karzon.** 1991. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest **88:**1026-33.

82. **Graham, B. S., T. R. Johnson, and R. S. Peebles.** 2000. Immune-mediated disease pathogenesis in respiratory syncytial virus infection. Immunopharmacology **48:**237-47.

83. **Groothuis, J. R.** 1994. The role of RSV neutralizing antibodies in the treatment and prevention of respiratory syncytial virus infection in high-risk children. Antiviral Res **23:**1-10.

84. **Groskreutz, D. J., M. M. Monick, L. S. Powers, T. O. Yarovinsky, D. C. Look, and G. W. Hunninghake.** 2006. Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. J Immunol **176:**1733-40.

85. **Groskreutz, D. J., M. M. Monick, T. O. Yarovinsky, L. S. Powers, D. E. Quelle, S. M. Varga, D. C. Look, and G. W. Hunninghake.** 2007. Respiratory syncytial virus decreases p53 protein to prolong survival of airway epithelial cells. J Immunol **179:**2741-7.

86. **Hall, C. B.** 1999. Respiratory syncytial virus: A continuing culprit and conundrum. J Pediatr **135:**2-7.

87. **Hall, C. B., C. E. Long, and K. C. Schnabel.** 2001. Respiratory syncytial virus infections in previously healthy working adults. Clin Infect Dis **33:**792-6.

88. **Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel.** 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis **163:**693-8.

89. **Hancock, G. E., P. W. Tebbey, C. A. Scheuer, K. S. Pryharski, K. M. Heers, and N. A. LaPierre.** 2003. Immune responses to the nonglycosylated ectodomain of respiratory syncytial virus attachment glycoprotein mediate pulmonary eosinophilia in inbred strains of mice with different MHC haplotypes. J Med Virol **70:**301-8.

90. **Harcourt, J., R. Alvarez, L. P. Jones, C. Henderson, L. J. Anderson, and R. A. Tripp.** 2006. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. J Immunol **176:**1600-8.

91. **Harrison, A. M., C. A. Bonville, H. F. Rosenberg, and J. B. Domachowske.** 1999. Respiratory syncytical virus-induced chemokine expression in the lower airways: eosinophil recruitment and degranulation. Am J Respir Crit Care Med **159:**1918-24.

92. **Henderson, F. W., A. M. Collier, W. A. Clyde, Jr., and F. W. Denny.** 1979. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N Engl J Med **300:**530-4.

93. **Hendricks, D. A., K. Baradaran, K. McIntosh, and J. L. Patterson.** 1987. APPEARANCE OF A SOLUBLE FORM OF THE G-PROTEIN OF RESPIRATORY SYNCYTIAL VIRUS IN FLUIDS OF INFECTED-CELLS. Journal of General Virology **68:**1705-1714.

94. **Hendricks, D. A., K. Baradaran, K. McIntosh, and J. L. Patterson.** 1987. Appearance of a soluble form of the G protein of respiratory syncytial virus in fluids of infected cells. J Gen Virol **68 (Pt 6):**1705-14.

95. **Hendricks, D. A., K. McIntosh, and J. L. Patterson.** 1988. Further characterization of the soluble form of the G glycoprotein of respiratory syncytial virus. J Virol **62:**2228-33.

96. **Hendry, R. M., J. C. Burns, E. E. Walsh, B. S. Graham, P. F. Wright, V. G. Hemming, W. J. Rodriguez, H. W. Kim, G. A. Prince, K. McIntosh, and et al.** 1988. Strain-specific serum antibody responses in infants undergoing primary infection with respiratory syncytial virus. J Infect Dis **157:**640-7.

97. **Hierholzer, J. C., and G. A. Tannock.** 1986. Respiratory syncytial virus: a review of the virus, its epidemiology, immune response and laboratory diagnosis. Aust Paediatr J **22:**77-82.

98. **Hoffman, S. J., F. R. Laham, and F. P. Polack.** 2004. Mechanisms of illness during respiratory syncytial virus infection: the lungs, the virus and the immune response. Microbes Infect **6:**767-72.

99. **Hussell, T., L. C. Spender, A. Georgiou, A. O'Garra, and P. J. Openshaw.** 1996. Th1 and Th2 cytokine induction in pulmonary T cells during infection with respiratory syncytial virus. J Gen Virol **77 (Pt 10):**2447-55.

100. **Jana, S., C. Chakraborty, S. Nandi, and J. K. Deb.** 2004. RNA interference: potential therapeutic targets. Appl Microbiol Biotechnol **65:**649-57.

101. **Johnson, P. R., Jr., R. A. Olmsted, G. A. Prince, B. R. Murphy, D. W. Alling, E. E. Walsh, and P. L. Collins.** 1987. Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: evaluation of the contributions of F and G glycoproteins to immunity. J Virol **61:**3163-6.

102. **Johnson, T. R., and B. S. Graham.** 1999. Secreted respiratory syncytial virus G glycoprotein induces interleukin-5 (IL-5), IL-13, and eosinophilia by an IL-4-independent mechanism. J Virol **73:**8485-95.

103. **Johnson, T. R., S. Hong, L. Van Kaer, Y. Koezuka, and B. S. Graham.** 2002. NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. J Virol **76:**4294-303.

104. **Joncas, J., L. Berthiaume, and V. Pavilanis.** 1969. The structure of the respiratory syncytial virus. Virology **38:**493-6.

105. **Kapikian, A. Z., J. A. Bell, F. M. Mastrota, K. M. Johnson, R. J. Huebner, and R. M. Chanock.** 1961. An outbreak of febrile illness and pneumonia associated with respiratory syncytial virus infection. Am J Hyg **74:**234-48.

106. **Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart.** 1969. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol **89:**405-21.

107. **Karron, R. A., D. A. Buonagurio, A. F. Georgiu, S. S. Whitehead, J. E. Adamus, M. L. Clements-Mann, D. O. Harris, V. B. Randolph, S. A. Udem, B. R. Murphy, and M. S. Sidhu.** 1997. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. Proc Natl Acad Sci U S A **94:**13961-6.

108. **Karron, R. A., P. F. Wright, R. B. Belshe, B. Thumar, R. Casey, F. Newman, F. P. Polack, V. B. Randolph, A. Deatly, J. Hackell, W. Gruber, B. R. Murphy, and P. L. Collins.** 2005. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. J Infect Dis **191:**1093-104.

109. **Karron, R. A., P. F. Wright, J. E. Crowe, Jr., M. L. Clements-Mann, J. Thompson, M. Makhene, R. Casey, and B. R. Murphy.** 1997. Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants, and children. J Infect Dis **176:**1428-36.

110. **Kerr, M. H., and J. Y. Paton.** 1999. Surfactant protein levels in severe respiratory syncytial virus infection. Am J Respir Crit Care Med **159:**1115-8.

111. **Khattar, S. K., A. S. Yunus, and S. K. Samal.** 2001. Mapping the domains on the phosphoprotein of bovine respiratory syncytial virus required for N-P and P-L interactions using a minigenome system. J Gen Virol **82:**775-9.

112. **Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. Jensen, and R. H. Parrott.** 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol **89:**422-34.

113. **Kimpen, J. L., and P. L. Ogra.** 1993. T cell redistribution kinetics after secondary infection of BALB/c mice with respiratory syncytial virus. Clin Exp Immunol **91:**78-82.

114. **Kneyber, M. C., and J. L. Kimpen.** 2004. Advances in respiratory syncytial virus vaccine development. Curr Opin Investig Drugs **5:**163-70.

115. **Kotelkin, A., E. A. Prikhod'ko, J. I. Cohen, P. L. Collins, and A. Bukreyev.** 2003. Respiratory syncytial virus infection sensitizes cells to apoptosis mediated by tumor necrosis factor-related apoptosis-inducing ligand. J Virol **77:**9156-72.

116. **Krusat, T., and H. J. Streckert.** 1997. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch Virol **142:**1247-54.

117. **Kulkarni, A. B., P. L. Collins, I. Bacik, J. W. Yewdell, J. R. Bennink, J. E. Crowe, Jr., and B. R. Murphy.** 1995. Cytotoxic T cells specific for a single peptide on the M2 protein of respiratory syncytial virus are the sole mediators of resistance induced by immunization with M2 encoded by a recombinant vaccinia virus. J Virol **69:**1261-4.

118. **Kuo, L., R. Fearns, and P. L. Collins.** 1996. The structurally diverse intergenic regions of respiratory syncytial virus do not modulate sequential transcription by a dicistronic minigenome. J Virol **70:**6143-50.

119. **Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson, and R. W. Finberg.** 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nature Immunology **1:**398-401.

120. **Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson, and R. W. Finberg.** 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol **1:**398-401.

121. **Langedijk, J. P., B. L. de Groot, H. J. Berendsen, and J. T. van Oirschot.** 1998. Structural homology of the central conserved region of the attachment protein G of respiratory syncytial virus with the fourth subdomain of 55-kDa tumor necrosis factor receptor. Virology **243:**293-302.

122. **Lawless-Delmedico, M. K., P. Sista, R. Sen, N. C. Moore, J. B. Antczak, J. M. White, R. J. Greene, K. C. Leanza, T. J. Matthews, and D. M. Lambert.** 2000. Heptad-repeat regions of respiratory syncytial virus F1 protein form a six-membered coiled-coil complex. Biochemistry **39:**11684-95.

123. **Levine, S., and R. Hamilton.** 1969. Kinetics of the respiratory syncytial virus growth cycle in HeLa cells. Arch Gesamte Virusforsch **28:**122-32.

124. **Levine, S., R. Klaiber-Franco, and P. R. Paradiso.** 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J Gen Virol **68 (Pt 9):**2521-4.

125. **Li, H. W., and S. W. Ding.** 2005. Antiviral silencing in animals. FEBS Lett.

126. **Lichtenstein, D. L., S. R. Roberts, G. W. Wertz, and L. A. Ball.** 1996. Definition and functional analysis of the signal/anchor domain of the human respiratory syncytial virus glycoprotein G. J Gen Virol **77 (Pt 1):**109-18.

127. **Lindemans, C. A., P. J. Coffer, I. M. Schellens, P. M. de Graaff, J. L. Kimpen, and L. Koenderman.** 2006. Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. J Immunol **176:**5529-37.

128. **Liu, P., M. Jamaluddin, K. Li, R. P. Garofalo, A. Casola, and A. R. Brasier.** 2007. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. J Virol **81:**1401-11.

129. **Loo, Y. M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M. A. Gill, A. Garcia-Sastre, M. G. Katze, and M. Gale, Jr.** 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J Virol **82:**335-45.

130. **Lopez, J. A., R. Bustos, C. Orvell, M. Berois, J. Arbiza, B. Garcia-Barreno, and J. A. Melero.** 1998. Antigenic structure of human respiratory syncytial virus fusion glycoprotein. J Virol **72:**6922-8.

131. **Low, K. W., T. Tan, K. Ng, B. H. Tan, and R. J. Sugrue.** 2008. The RSV F and G glycoproteins interact to form a complex on the surface of infected cells. Biochem Biophys Res Commun **366:**308-13.

132. **Lowther, S. A., D. K. Shay, R. C. Holman, M. J. Clarke, S. F. Kaufman, and L. J. Anderson.** 2000. Bronchiolitis-associated hospitalizations among American Indian and Alaska Native children. Pediatr Infect Dis J **19:**11-7.

133. **Lu, P. Y., F. Xie, and M. C. Woodle.** 2005. In vivo application of RNA interference: from functional genomics to therapeutics. Adv Genet **54:**117-42.

134. **Martinez, F. D.** 2003. Respiratory syncytial virus bronchiolitis and the pathogenesis of childhood asthma. Pediatr Infect Dis J **22:**S76-82.

135. **Martinez, I., J. Dopazo, and J. A. Melero.** 1997. Antigenic structure of the human respiratory syncytial virus G glycoprotein and relevance of hypermutation events for the generation of antigenic variants. Journal of General Virology **78:**2419-2429.

136. **Martinez, I., L. Lombardia, B. Garcia-Barreno, O. Dominguez, and J. A. Melero.** 2007. Distinct gene subsets are induced at different time points after human respiratory syncytial virus infection of A549 cells. J Gen Virol **88:**570-81.

137. **Matthews, S. P., J. S. Tregoning, A. J. Coyle, T. Hussell, and P. J. Openshaw.** 2005. Role of CCL11 in eosinophilic lung disease during respiratory syncytial virus infection. J Virol **79:**2050-7.

138. **Medzhitov, R., and C. A. Janeway, Jr.** 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell **91:**295-8.

139. **Melero, J. A., B. Garcia-Barreno, I. Martinez, C. R. Pringle, and P. A. Cane.** 1997. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. J Gen Virol **78 (Pt 10):**2411-8.

140. **Miller, A. L., T. L. Bowlin, and N. W. Lukacs.** 2004. Respiratory syncytial virus-induced chemokine production: linking viral replication to chemokine production in vitro and in vivo. J Infect Dis **189:**1419-30.

141. **Mills, J. t., J. E. Van Kirk, P. F. Wright, and R. M. Chanock.** 1971. Experimental respiratory syncytial virus infection of adults. Possible mechanisms of resistance to infection and illness. J Immunol **107:**123-30.

142. **Mobbs, K. J., R. L. Smyth, U. O'Hea, D. Ashby, P. Ritson, and C. A. Hart.** 2002. Cytokines in severe respiratory syncytial virus bronchiolitis. Pediatr Pulmonol **33:**449-52.

143. **Monick, M. M., K. Cameron, J. Staber, L. S. Powers, T. O. Yarovinsky, J. G. Koland, and G. W. Hunninghake.** 2005. Activation of the epidermal growth factor receptor by respiratory syncytial virus results in increased inflammation and delayed apoptosis. J Biol Chem **280:**2147-58.

144. **Monick, M. M., T. O. Yarovinsky, L. S. Powers, N. S. Butler, A. B. Carter, G. Gudmundsson, and G. W. Hunninghake.** 2003. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. J Biol Chem **278:**53035-44.

145. **Moore, M. L., and R. S. Peebles, Jr.** 2006. Respiratory syncytial virus disease mechanisms implicated by human, animal model, and in vitro data facilitate vaccine strategies and new therapeutics. Pharmacol Ther **112:**405-24.

146. **Moretta, A., E. Marcenaro, S. Parolini, G. Ferlazzo, and L. Moretta.** 2008. NK cells at the interface between innate and adaptive immunity. Cell Death Differ **15:**226-33.

147. **Moretta, L., G. Ferlazzo, C. Bottino, M. Vitale, D. Pende, M. C. Mingari, and A. Moretta.** 2006. Effector and regulatory events during natural killer-dendritic cell interactions. Immunol Rev **214:**219-28.

148. **Muelenaer, P. M., F. W. Henderson, V. G. Hemming, E. E. Walsh, L. J. Anderson, G. A. Prince, and B. R. Murphy.** 1991. Group-specific serum antibody responses in children with primary and recurrent respiratory syncytial virus infections. J Infect Dis **164:**15-21.

149. **Mufson, M. A., C. Orvell, B. Rafnar, and E. Norrby.** 1985. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol **66 (Pt 10):**2111-24.

150. **Murawski, M. R., G. N. Bowen, A. M. Cerny, L. J. Anderson, L. M. Haynes, R. A. Tripp, E. A. Kurt-Jones, and R. W. Finberg.** 2009. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. J Virol **83:**1492-500.

151. **Murawski, M. R., G. N. Bowen, A. M. Cerny, L. J. Anderson, L. M. Haynes, R. A. Tripp, E. A. Kurt-Jones, and R. W. Finberg.** 2008. RSV Activates Innate Immunity through Toll-like Receptor 2. J Virol.

152. **Murphy, B. R., D. W. Alling, M. H. Snyder, E. E. Walsh, G. A. Prince, R. M. Chanock, V. G. Hemming, W. J. Rodriguez, H. W. Kim, B. S. Graham, and et al.** 1986. Effect of age and preexisting antibody on serum antibody response of infants and children to the F and G glycoproteins during respiratory syncytial virus infection. J Clin Microbiol **24:**894-8.

153. **Murphy, L. B., C. Loney, J. Murray, D. Bhella, P. Ashton, and R. P. Yeo.** 2003. Investigations into the amino-terminal domain of the respiratory syncytial virus nucleocapsid protein reveal elements important for nucleocapsid formation and interaction with the phosphoprotein. Virology **307:**143-53.

154. **Norrby, E., H. Marusyk, and C. Orvell.** 1970. Ultrastructural studies of the multiplication of RS (respiratory syncytial) virus. Acta Pathol Microbiol Scand [B] Microbiol Immunol **78:**268.

155. **Olson, M. R., S. M. Hartwig, and S. M. Varga.** 2008. The number of respiratory syncytial virus (RSV)-specific memory CD8 T cells in the lung is critical for their ability to inhibit RSV vaccine-enhanced pulmonary eosinophilia. J Immunol **181:**7958-68.

156. **Openshaw, P. J.** 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am J Respir Crit Care Med **152:**S59-62.

157. **Openshaw, P. J.** 2002. Potential therapeutic implications of new insights into respiratory syncytial virus disease. Respir Res **3 Suppl 1:**S15-20.

158. **Openshaw, P. J., K. Anderson, G. W. Wertz, and B. A. Askonas.** 1990. The 22,000-kilodalton protein of respiratory syncytial virus is a major target for Kd-restricted cytotoxic T lymphocytes from mice primed by infection. J Virol **64:**1683-9.

159. **Ostler, T., T. Hussell, C. D. Surh, P. Openshaw, and S. Ehl.** 2001. Long-term persistence and reactivation of T cell memory in the lung of mice infected with respiratory syncytial virus. Eur J Immunol **31:**2574-82.

160. **Palomo, C., P. A. Cane, and J. A. Melero.** 2000. Evaluation of the antibody specificities of human convalescent-phase sera against the attachment (G) protein of human respiratory syncytial virus: influence of strain variation and carbohydrate side chains. J Med Virol **60:**468-74.

161. **Palomo, C., B. Garcia-Barreno, C. Penas, and J. A. Melero.** 1991. The G protein of human respiratory syncytial virus: significance of carbohydrate side-chains and the C-terminal end to its antigenicity. J Gen Virol **72 (Pt 3):**669-75.

162. **Peret, T. C., C. B. Hall, K. C. Schnabel, J. A. Golub, and L. J. Anderson.** 1998. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J Gen Virol **79 (Pt 9):**2221-9.

163. **Polack, F. P., and R. A. Karron.** 2004. The future of respiratory syncytial virus vaccine development. Pediatr Infect Dis J **23:**S65-73.

164. **Preston, F. M., P. L. Beier, and J. H. Pope.** 1995. Identification of the respiratory syncytial virus-induced immunosuppressive factor produced by human peripheral blood mononuclear cells in vitro as interferon-alpha. J Infect Dis **172:**919-26.

165. **Pribul, P. K., J. Harker, B. Wang, H. Wang, J. S. Tregoning, J. Schwarze, and P. J. Openshaw.** 2008. Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. J Virol.

166. **Prince, G. A., R. L. Horswood, and R. M. Chanock.** 1985. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J Virol **55:**517-20.

167. **Reed, J. L., Y. A. Brewah, T. Delaney, T. Welliver, T. Burwell, E. Benjamin, E. Kuta, A. Kozhich, L. McKinney, J. Suzich, P. A. Kiener, L. Avendano, L. Velozo, A. Humbles, R. C. Welliver Sr, and A. J. Coyle.** 2008. Macrophage Impairment Underlies Airway Occlusion in Primary Respiratory Syncytial Virus Bronchiolitis. J Infect Dis.

168. **Roberts, S. R., D. Lichtenstein, L. A. Ball, and G. W. Wertz.** 1994. The membrane-associated and secreted forms of the respiratory syncytial virus attachment glycoprotein G are synthesized from alternative initiation codons. J Virol **68:**4538-46.

169. **Roman, M., W. J. Calhoun, K. L. Hinton, L. F. Avendano, V. Simon, A. M. Escobar, A. Gaggero, and P. V. Diaz.** 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med **156:**190-5.

170. **Routledge, E. G., M. M. Willcocks, L. Morgan, A. C. Samson, R. Scott, and G. L. Toms.** 1987. Expression of the respiratory syncytial virus 22K protein on the surface of infected HeLa cells. J Gen Virol **68 (Pt 4):**1217-22.

171. **Rudd, B. D., E. Burstein, C. S. Duckett, X. Li, and N. W. Lukacs.** 2005. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. J Virol **79:**3350-7.

172. **Rudd, B. D., J. J. Smit, R. A. Flavell, L. Alexopoulou, M. A. Schaller, A. Gruber, A. A. Berlin, and N. W. Lukacs.** 2006. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. J Immunol **176:**1937-42.

173. **Rueda, P., C. Palomo, B. Garciabarreno, and J. A. Melero.** 1995. THE 3 C-TERMINAL RESIDUES OF HUMAN RESPIRATORY SYNCYTIAL VIRUS G-GLYCOPROTEIN (LONG STRAIN) ARE ESSENTIAL FOR INTEGRITY OF

MULTIPLE EPITOPES DISTINGUISHABLE BY ANTIIDIOTYPIC ANTIBODIES. Viral Immunology **8:**37-46.

174. **Rutigliano, J. A., M. T. Rock, A. K. Johnson, J. E. Crowe, Jr., and B. S. Graham.** 2005. Identification of an H-2D(b)-restricted CD8+ cytotoxic T lymphocyte epitope in the matrix protein of respiratory syncytial virus. Virology **337:**335-43.

175. **Ryther, R. C., A. S. Flynt, J. A. Phillips, 3rd, and J. G. Patton.** 2005. siRNA therapeutics: big potential from small RNAs. Gene Ther **12:**5-11.

176. **Sakurai, H., R. A. Williamson, J. E. Crowe, J. A. Beeler, P. Poignard, R. B. Bastidas, R. M. Chanock, and D. R. Burton.** 1999. Human antibody responses to mature and immature forms of viral envelope in respiratory syncytial virus infection: significance for subunit vaccines. J Virol **73:**2956-62.

177. **Salkind, A. R., D. O. McCarthy, J. E. Nichols, F. M. Domurat, E. E. Walsh, and N. J. Roberts, Jr.** 1991. Interleukin-1-inhibitor activity induced by respiratory syncytial virus: abrogation of virus-specific and alternate human lymphocyte proliferative responses. J Infect Dis **163:**71-7.

178. **Sandy, P., A. Ventura, and T. Jacks.** 2005. Mammalian RNAi: a practical guide. Biotechniques **39:**215-24.

179. **Schelender J, W. G., Conzelmann K-K.** 2000b. Contact inhibition of PBL proliferation by respiratory syncytial virus(RSV) fusion (F) protein, p. 200, 11th International Conference on Negative Strand Viruses.

180. **Scherer, L., and J. J. Rossi.** 2004. Recent applications of RNAi in mammalian systems. Curr Pharm Biotechnol **5:**355-60.

181. **Scherr, M., and M. Eder.** 2004. RNAi in functional genomics. Curr Opin Mol Ther **6:**129-35.

182. **Schlender, J., B. Bossert, U. Buchholz, and K. K. Conzelmann.** 2000. Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize alpha/beta interferon-induced antiviral response. J Virol **74:**8234-42.

183. **Schlender, J., V. Hornung, S. Finke, M. Gunthner-Biller, S. Marozin, K. Brzozka, S. Moghim, S. Endres, G. Hartmann, and K. K. Conzelmann.** 2005. Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. J Virol **79:**5507-15.

184. **Shankar, P., N. Manjunath, and J. Lieberman.** 2005. The prospect of silencing disease using RNA interference. Jama **293:**1367-73.

185. **Shay, D. K., R. C. Holman, R. D. Newman, L. L. Liu, J. W. Stout, and L. J. Anderson.** 1999. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA **282:**1440-6.

186. **Shingai, M., M. Azuma, T. Ebihara, M. Sasai, K. Funami, M. Ayata, H. Ogura, H. Tsutsumi, M. Matsumoto, and T. Seya.** 2008. Soluble G protein of respiratory syncytial virus inhibits Toll-like receptor 3/4-mediated IFN-beta induction. Int Immunol **20:**1169-80.

187. **Siegrist, C. A.** 2003. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. Vaccine **21:**3406-12.

188. **Siegrist, C. A., C. Barrios, X. Martinez, C. Brandt, M. Berney, M. Cordova, J. Kovarik, and P. H. Lambert.** 1998. Influence of maternal antibodies on vaccine responses: inhibition of antibody but not T cell responses allows successful early prime-boost strategies in mice. Eur J Immunol **28:**4138-48.

189. **Simoes, E. A.** 1999. Respiratory syncytial virus infection. Lancet **354:**847-52.

190. **Smit, J. J., D. M. Lindell, L. Boon, M. Kool, B. N. Lambrecht, and N. W. Lukacs.** 2008. The Balance between Plasmacytoid DC versus Conventional DC Determines Pulmonary Immunity to Virus Infections. PLoS ONE **3:**e1720.

191. **Smit, J. J., B. D. Rudd, and N. W. Lukacs.** 2006. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. J Exp Med **203:**1153-9.

192. **Spann, K. M., K. C. Tran, B. Chi, R. L. Rabin, and P. L. Collins.** 2004. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. J Virol **78:**4363-9.

193. **Spann, K. M., K. C. Tran, and P. L. Collins.** 2005. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J Virol **79:**5353-62.

194. **Staat, M. A.** 2002. Respiratory syncytial virus infections in children. Semin Respir Infect **17:**15-20.

195. **Stec, D. S., M. G. Hill, 3rd, and P. L. Collins.** 1991. Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of nonsegmented negative-strand viruses. Virology **183:**273-87.

196. **Takaku, H.** 2004. Gene silencing of HIV-1 by RNA interference. Antivir Chem Chemother **15:**57-65.

197. **Tan, F. L., and J. Q. Yin.** 2004. RNAi, a new therapeutic strategy against viral infection. Cell Res **14:**460-6.

198. **Taub, D. D., J. R. Ortaldo, S. M. Turcovski-Corrales, M. L. Key, D. L. Longo, and W. J. Murphy.** 1996. Beta chemokines costimulate lymphocyte cytolysis, proliferation, and lymphokine production. J Leukoc Biol **59:**81-9.

199. **Taylor, G., E. J. Stott, and A. J. Hayle.** 1985. Cytotoxic lymphocytes in the lungs of mice infected with respiratory syncytial virus. J Gen Virol **66 (Pt 12):**2533-8.

200. **Tebbey, P. W., M. Hagen, and G. E. Hancock.** 1998. Atypical pulmonary eosinophilia is mediated by a specific amino acid sequence of the attachment (G) protein of respiratory syncytial virus. J Exp Med **188:**1967-72.

201. **Techaarpornkul, S., P. L. Collins, and M. E. Peeples.** 2002. Respiratory syncytial virus with the fusion protein as its only viral glycoprotein is less dependent on cellular glycosaminoglycans for attachment than complete virus. Virology **294:**296-304.

202. **Tekkanat, K. K., H. Maassab, A. Miller, A. A. Berlin, S. L. Kunkel, and N. W. Lukacs.** 2002. RANTES (CCL5) production during primary respiratory syncytial virus infection exacerbates airway disease. Eur J Immunol **32:**3276-84.

203. **Teng, M. N., S. S. Whitehead, and P. L. Collins.** 2001. Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication in vitro and in vivo. Virology **289:**283-96.

204. **Tripp, R. A.** 2004. Pathogenesis of respiratory syncytial virus infection. Viral Immunol **17:**165-81.

205. **Tripp, R. A., L. P. Jones, L. M. Haynes, H. Zheng, P. M. Murphy, and L. J. Anderson.** 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol **2:**732-8.

206. **Tripp, R. A., D. Moore, A. t. Barskey, L. Jones, C. Moscatiello, H. Keyserling, and L. J. Anderson.** 2002. Peripheral blood mononuclear cells from infants hospitalized because of respiratory syncytial virus infection express T helper-1 and T helper-2 cytokines and CC chemokine messenger RNA. J Infect Dis **185:**1388-94.

207. **Tripp, R. A., D. Moore, L. Jones, W. Sullender, J. Winter, and L. J. Anderson.** 1999. Respiratory syncytial virus G and/or SH protein alters Th1 cytokines, natural killer cells, and neutrophils responding to pulmonary infection in BALB/c mice. J Virol **73:**7099-107.

208. **Tripp, R. A., C. Oshansky, and R. Alvarez.** 2005. Cytokines and respiratory syncytial virus infection. Proc Am Thorac Soc **2:**147-9.

209. **Trudel, M., F. Nadon, C. Seguin, and H. Binz.** 1991. Protection of BALB/c mice from respiratory syncytial virus infection by immunization with a synthetic peptide derived from the G glycoprotein. Virology **185:**749-57.

210. **Valarcher, J. F., J. Furze, S. Wyld, R. Cook, K. K. Conzelmann, and G. Taylor.** 2003. Role of alpha/beta interferons in the attenuation and immunogenicity of recombinant bovine respiratory syncytial viruses lacking NS proteins. J Virol **77:**8426-39.

211. **Van Schaik, S. M., I. Vargas, R. C. Welliver, and G. Enhorning.** 1997. Surfactant dysfunction develops in BALB/c mice infected with respiratory syncytial virus. Pediatr Res **42:**169-73.

212. **van Schaik, S. M., R. C. Welliver, and J. L. Kimpen.** 2000. Novel pathways in the pathogenesis of respiratory syncytial virus disease. Pediatr Pulmonol **30:**131-8.

213. **Varga, S. M., X. Wang, R. M. Welsh, and T. J. Braciale.** 2001. Immunopathology in RSV infection is mediated by a discrete oligoclonal subset of antigen-specific CD4(+) T cells. Immunity **15:**637-46.

214. **Vijaya, S., N. Elango, F. Zavala, and B. Moss.** 1988. Transport to the cell surface of a peptide sequence attached to the truncated C terminus of an N-terminally anchored integral membrane protein. Mol Cell Biol **8:**1709-14.

215. **Walsh, E. E., and J. Hruska.** 1983. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. J Virol **47:**171-7.

216. **Wang, H., N. Peters, and J. Schwarze.** 2006. Plasmacytoid dendritic cells limit viral replication, pulmonary inflammation, and airway hyperresponsiveness in respiratory syncytial virus infection. J Immunol **177:**6263-70.

217. **Wang, H., Z. Su, and J. Schwarze.** 2008. Healthy, but not RSV-infected, lung epithelial cells profoundly inhibit T-cell activation. Thorax.

218. **Wertz, G. W., P. L. Collins, Y. Huang, C. Gruber, S. Levine, and L. A. Ball.** 1985. Nucleotide sequence of the G protein gene of human respiratory syncytial virus reveals an unusual type of viral membrane protein. Proc Natl Acad Sci U S A **82:**4075-9.

219. **Wilson, J., K. Rowlands, K. Rockett, C. Moore, E. Lockhart, M. Sharland, D. Kwiatkowski, and J. Hull.** 2005. Genetic variation at the IL10 gene locus is associated with severity of respiratory syncytial virus bronchiolitis. J Infect Dis **191:**1705-9.

220. **Wissinger, E. L., W. W. Stevens, S. M. Varga, and T. J. Braciale.** 2008. Proliferative expansion and acquisition of effector activity by memory CD4+ T cells in the lungs following pulmonary virus infection. J Immunol **180:**2957-66.

221. **Wright, P. F., R. A. Karron, R. B. Belshe, J. Thompson, J. E. Crowe, Jr., T. G. Boyce, L. L. Halburnt, G. W. Reed, S. S. Whitehead, E. L. Anderson, A. E. Wittek,**

R. Casey, M. Eichelberger, B. Thumar, V. B. Randolph, S. A. Udem, R. M. Chanock, and B. R. Murphy. 2000. Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy. J Infect Dis **182:**1331-42.

222. **Yeo, S. J., Y. J. Yun, M. A. Lyu, S. Y. Woo, E. R. Woo, S. J. Kim, H. J. Lee, H. K. Park, and Y. H. Kook.** 2002. Respiratory syncytial virus infection induces matrix metalloproteinase-9 expression in epithelial cells. Arch Virol **147:**229-42.

223. **Yoshimura, A., T. Naka, and M. Kubo.** 2007. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol **7:**454-65.

224. **Zhao, D., D. Peng, L. Li, Q. Zhang, and C. Zhang.** 2008. Inhibition of G1P3 expression found in the differential display study on respiratory syncytial virus infection. Virol J **5:**114.

CHAPTER 3

RNA INTERFERENCE INHIBITS RESPIRATORY SYNCYTIAL VIRUS REPLICATION AND DISEASE PATHOGENESIS WITHOUT INHIBITING PRIMING OF THE MEMORY IMMUNE RESPONSE[1]

---

[1]Wenliang Zhang[*] and Ralph A. Tripp[*#], J Virol 82(24):12221-31, 2008

Reprinted here with permission of publisher

Abstract

Respiratory syncytial virus (RSV) is a major cause of morbidity in infants, young children, and the elderly worldwide. Currently there is no effective vaccine and anti-viral drugs to control infection are limited. RNA interference (RNAi) is a powerful tool amenable to development of anti-viral drugs. Using small interfering RNA (siRNA) targeting the RSV P gene (siRNA-P), RSV replication can be silenced both in vitro and in a BALB/c model of RSV infection. In this study, we examine the effect of siRNA prophylaxis on the primary and memory immune response to RSV infection in mice. We show that mice prophylactically treated with siRNA-P to decrease but not eliminate RSV replication exhibit reduced pulmonary inflammation and lung pathogenesis, and produce a robust anti-RSV memory response when subsequently challenged with RSV. The pulmonary T cell memory response was characterized by high numbers of $CD44^{hi}CD62L^{lo}$ $CD4^{+}$ and $CD8^{+}$ T cells, M2 peptide-$tetramer^{+}$ $CD8^{+}$ T cells expressing IFNγ, and a RSV-specific antibody response. The results support the hypothesis that siRNAs can be developed as effective anti-viral drugs that can be used to reduce the viral load and parameters of pathogenesis without limiting the induction of the memory immune response.

Introduction

Respiratory syncytial virus (RSV) is an enveloped negative-strand RNA virus belonging to the Paramyxoviridae family. RSV is a primary cause of morbidity and some mortality in infants, young children, the elderly, and the immune suppressed causing bronchiolitis and pneumonia-related illness (20, 23, 68). In addition, RSV infection has also been linked to the development or exacerbation of airway hyperresponsiveness in children (5, 18, 44), thus there is a substantial need for effective vaccines and anti-viral drugs. To date all RSV vaccination strategies have proven to be ineffective (23, 47). Compounding issues, natural RSV infection

does not lead to sustained protective immunity as individuals may be repeatedly infected with the same or different strains (7, 52). The features that contribute to lack of durable immunity are not fully understood, but antigenic variation as a mechanism is unlikely. Seasonal epidemics linked to RSV infections do not appear to have a major role in susceptibility to reinfection as the two major viral surface proteins that are the targets of neutralizing antibody, i.e. attachment (G) and fusion (F) proteins, do not exhibit dramatic seasonal variation such as observed for influenza virus (10, 29). However, these proteins have been shown to modify aspects of the immune response, particularly the G protein which has been shown to inhibit fractalkine-mediated responses, alter trafficking of $CX3CR1^+$ cells immune cells, modify the magnitude and cadence of cytokine and chemokine expression, affect TCR Vβ usage by $CD4^+$ T cells, and affect the interface with the neuro-immune system through induction of the proinflammatory tachykinin, substance P (31, 59-66). It is likely that these and other mechanisms contribute to immune dysregulation that may facilitate virus replication and/or contribute to persistence of RSV infection (5, 72).

Numerous studies in BALB/c mice have shown that cell mediated immunity is important in the resolution of RSV infection, a feature mediated by both $CD4^+$ and $CD8^+$ T cells (49). The RSV M2 protein has been identified as a major H-2^d-restricted CTL target (15, 35, 36). Consistent with the lack of durable immunologic memory generated after RSV infection, M2-specific effector $CD8^+$ T cells isolated from the lungs of infected mice have been shown to have a reduced capacity to express IFNγ, whereas similar effector $CD8^+$ T cells in the spleen have no deficit in IFNγ expression (8). Intriguingly, a rapid loss in the frequency of RSV-specific memory $CD8^+$ T cells occurs in the lungs of the infected mice during the resolution phase of infection, a finding consistent with the notion that natural RSV infection does not lead to

sustained protective immunity (12, 13, 55). Thus, converging lines of evidence suggest that RSV protein expression contributes to the dysregulation of immune a function, a feature that negatively affects the development of immune memory.

Since safe and effective RSV vaccines are not available, disease management has focused on passive immunotherapy for high-risk patients. Palivizumab, a humanized IgG monoclonal antibody targeting the RSV F protein is currently used as a passive immunoprophylaxis (14, 24). However, this treatment has modest prophylactic efficacy, highlighting the need for an actual antiviral that could be used as a treatment. RNA interference (RNAi) is a mechanism that inhibits gene expression at the stage of translation by hindering the transcription of specific genes (30, 43, 45). The process is mediated by small interfering RNA (siRNA) that has complementary nucleotide sequence to the targeted RNA strand. The siRNAs are guided to their cognate targets by components of the RNA-induced silencing complex (RISC) where they may cleave the target to prevent translation into protein (58). RNAi is a compelling tool for rationalize drug design, and is being tested as a prophylactic and therapeutic antiviral agent for a range of viruses including HIV, hepatitis, as well as RSV (3, 4, 32, 42, 56). Given the pace of RNAi applications and demonstrated efficacy, it is likely that RNAi-based therapeutics will evolve to be a major therapeutic modality for antiviral treatment of numerous viruses.

RSV has been successfully targeted by siRNA (2-4, 6). Intranasal delivery of an in vitro-active siRNA directed at the P gene of RSV significantly inhibits RSV replication (6). In these studies, siRNA prophylactically delivered to mice 4 hours before RSV infection reduced lung virus titers and prevented pulmonary pathology. When RSV-infected mice were treated therapeutically with the drug, the level of antiviral efficacy was diminished but lung virus titers were still reduced. Since RSV replicates almost exclusively in the respiratory epithelium of

humans (71), siRNA antiviral drugs can be directly administered using topical or aerosol delivery methods. Although the preliminary evidence suggests siRNA drugs targeting RSV may be beneficial, unfortunately nothing is known about the effects of siRNA treatment on the memory response to viral challenge.

In this study, we examined the effect of siRNA prophylaxis on the primary and memory immune response to RSV infection in BALB/c mice. Mice were treated prophylactically with siRNA targeting the RSV P gene to reduce but not eliminate RSV replication so that the relationship between virus load, disease pathogenesis and immunity could be evaluated. We show that siRNA drugs that reduce RSV replication effectively prevent lung disease pathogenesis and allow for robust anti-RSV memory immune responses.

Materials and Methods

Small inhibitory RNA (siRNA). The wild type (WT) siRNA corresponding to the sequence AAGCCCTATAACATCAAATTCAA of the P gene mRNA (6) of RSV strain A2, and a mismatch (MM) control siRNA of similar content was used in the studies. Each strand of the siRNA was 21 nucleotides long and contained 3'-terminal dTdT extensions. All siRNAs were commercially synthesized (Dharmacon ThermoFisher). The WT or MM siRNAs were diluted in PBS (100 nM) and intranasally instilled in mice.

Virus Infection. Vero cells (African green monkey kidney cells) were grown in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal bovine sera (Hyclone; DMEM-10%). Respiratory syncytial virus strain A2 (RSV) was propagated in Vero cells as previously described (64). Briefly, semi-confluent Vero cells were prepared and washed with phosphate buffered saline (PBS). RSV was diluted in DMEM and the cells infected at a multiplicity of infection (MOI) of 1. The virus was allowed to adsorb for 2 hour at 37^{o}C after which DMEM-

10% was added and the cells incubated at 37^{o}C for 4 days. At day 4 post-infection (pi), the virus was recovered by removing the cell culture supernatant, freeze-thawing the infected cells, and centrifuging the cell lysate to remove debris and recover the virus from the cell lysate supernatant.

Mice, treatment and infections. Four-to-six week old specific-pathogen free female BALB/c mice were purchased from Charles River Laboratories, housed in microisolator cages, and fed sterilized water and food *ad libitum*. The studies were reviewed and approved by the university institutional review committee. Mice were prophylactically treated for 12h by topical instillation of either WT or MM siRNA (2 mg/kg) or PBS prior to infection. In primary immune response studies, the treated mice were challenged with 10^6 PFU of RSV strain A2 (RSV/A2) diluted in PBS (GIBCO BRL), and 10 mice/group/day harvested at days 0, 2, 4, 6 or 8 pi. A portion of these mice were rested for three weeks post-infection, and for memory immune response studies, were i.n. challenged with 10^6 PFU of RSV strain A/Long (10 mice/group/day) and harvested at days 0, 2, 4, 6, 8 or 10 pi. The organs were collected from primary and memory immune mice following anesthetization and exsanguinations mediated by severing the right caudal artery. The blood was collected for sera, and the bronchoalveolar leukocytes (BAL) collected by lavaging the lungs of the mice 3x with 1 ml of PBS. The lungs from three mice/group/day not collected for BAL were harvested for histopathology, or determination of virus titer determined by immunostaining plaque assay on Vero cells as previously described (64).

Flow cytometry. The percent positive B220, CD3, CD4, CD8, CD11b, CD44, CD62L, DX5, and RB6-8C5 cell subsets were determined for BAL cells by flow cytometry. Cells were blocked with 10% normal mouse sera (Jackson Laboratories) in flow buffer (PBS+ 1% BSA), and then stained with the appropriate combinations of FITC- or PE-labeled anti-B cell (RA3-6B2), anti-

CD3 (2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD44 (KM114), anti-CD62L (DREG-56), anti-pan NK cell (DX5), anti-pan neutrophil (RB6-8C5) or mouse isotype antibody control (all from BD Pharmingen, San Diego, CA) as previously described (64). For intracellular cytokine staining, BAL cells were incubated for 3 h in the presence of 10 μg/ml brefeldin A (BD Pharmingen). After incubation, the cells were washed with flow buffer and blocked with 10% normal mouse sera (Jackson Laboratories) in flow buffer. After blocking for 15 min, the cells were washed with flow buffer and incubated with optimal concentrations of anti-CD4 PerCP-Cy5.5 (BD Pharmingen) or CD8 Pe-Cy5.5 (BD Pharmingen) for 30 min at 4°C. Cells were then washed with flow buffer and fixed with 4% paraformaldehyde. Cells were then washed in permeabilization buffer (BD Pharmingen) and stained with an optimal concentration of anti-IFN-γ-PE (clone XMG1.2; BD Pharmingen) or anti-IL-6 (clone; BD Pharmingen) antibody diluted in permeabilization bufferCells were washed and analyzed on a BD LSRII flow cytometer using FACSDiva software (Becton-Dickinson, Mountain View, CA) from >10,000 lymphocyte gated events.

RSV-specific antibody response. Antibody titer and isotypes of the sera samples were determined as previously published (57). Briefly, 96-well high binding ELISA plates (Corning Costar, Corning, NY) were coated with 1 μg of RSV/A2 (10^6 PFU/ml) or 1 μg of uninfected Vero cell lysate, and blocked with blocking buffer containing PBS, 0.3% Tween-20 (Sigma) and 0.01 M EDTA buffer (Sigma)(pH 7.0). Dilutions of the sera were made in blocking buffer, added to the wells, and incubated for 1 h at 37^{o}C. After five washes in PBS containing 0.1% Tween-20, biotinylated goat anti-mouse IgG (BD Pharmingen) was added to the wells and the plates incubated for 1 h at 37^{o}C. After washing, an appropriate dilution of streptavidin conjugated to

horseradish peroxidase (Zymed Laboratories, Inc., San Francisco, CA) was added to the wells and plates were incubated for 30 min at room temperature. Following removal of the unbound streptavidin by washing, peroxidase substrate (2,2 '-azino-di[3-ethyl-benzthiazoline sulfonate]) (Kirkegaard and Perry Laboratories, Gaithersberg, MD) was added to the wells for 20 min. The reaction was stopped with 1% SDS. The determination of anti-RSV titers was determined by optical densities at 410 nm (reference 630 nm) on a Tecan ELISA plate reader (Tecan, Research Triangle Park, NC). Data are presented as the endpoint titer calculated as the reciprocal of the geometric mean of the dilution that resulted in an OD410 reading of 0.03. Isotypes determination kits (ThermoScientific, Rockford, IL) were used for antibody isotyping as described by the manufacturer.

RSV M2 tetramer staining. BAL cells suspensions were blocked with purified anti-FcγRII/III monoclonal antibody (BD Pharmingen) for 30 min. Cells were washed with flow buffer and incubated with optimal concentrations of anti-CD8 FITC antibody (BD Pharmingen). Cells were washed and incubated with optimal concentrations of $M2_{82–90}$- specific H-$2k^d$ allophycocyanin-conjugated tetramers (obtained from the Emory University Tetramer Core Facility, Atlanta, GA) for 45 min at 4°C. After tetramer staining, cells were washed twice with flow buffer and analyzed on a BD LSRII flow cytometer using FACSDiva software (Becton-Dickinson, Mountain View, CA) from >10,000 lymphocyte gated events.

Histopathology. Histopathological examination was performed for lungs from siRNA-treated and untreated mice infected with RSV. Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to light microscopy observation. Multiple sections from each tissue block were analyzed under light microscopy.

Statistics. For statistical evaluation between two treatments, a *t*-test for unpaired samples was used to compare the responses between WT and MM siRNA treated mice. Values with $p<0.05$ or $p<0.01$ were considered significant. The Kruskal-Wallis test (Prism Graph Pad, La Jolla, CA) was used to compare the medians between the three treatment groups where the significance was set at $P < 0.05$.

Results

Prophylactic siRNA treatment reduces virus lung titers and lung pathogenesis.

Previous prophylactic and therapeutic studies have shown that siRNAs targeting the P gene can reduce RSV replication (6). Using the same siRNAs targeting the P gene (WT), a scrambled mismatch (MM) siRNA control, or PBS, the level of lung virus replication was determined. Mice prophylactically treated by intranasal (i.n.) inoculation with 200, 100 or 50 nM of siRNA for 12h prior to RSV infection had reduced lung virus titers at day 4 pi, an effect that was dose-dependent, in contrast to MM siRNA or PBS treated mice where no effect on virus titer was observed (Figure 3.1 A).

To evaluate in vivo efficacy of siRNA prophylaxis, mice were i.n. treated with 100 nM (2 mg/kg) WT or MM siRNA or PBS vehicle and 12h post-treatment i.n. infected with 10^6 pfu RSV/A2 (Figure 1B). Prophylactic treatment with WT siRNA reduced virus titers at all time points examined compared to MM siRNA or PBS-treated mice, and at days 4 and 6 pi, WT siRNA treated mice had significantly ($p<0.05$) reduced virus titers compared to MM siRNA or PBS-treated mice. No substantial lung histopathology was detected WT siRNA-treated mice at any time point examined. MM siRNA and PBS treated mice did not show substantial histopathology until day 6 pi. For comparison, the histopathology at day 6 pi is shown for WT siRNA treated mice (Figure 3.1Ci), PBS-treated mice (Figure 3.1Cii) and MM siRNA-treated

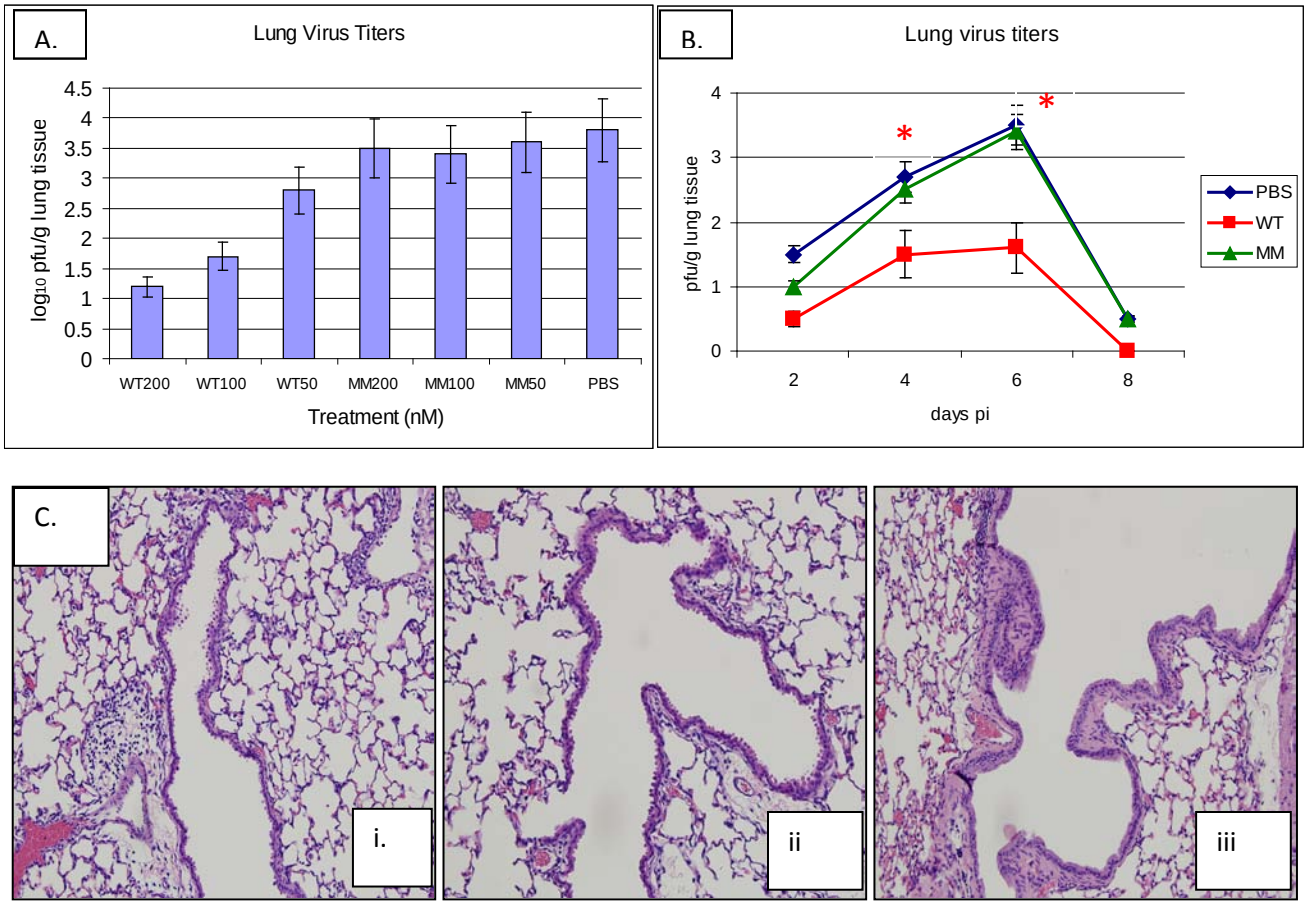


Figure 3.1. Prophylactic siRNA treatment reduces virus lung titers. A) Mice were prophylactically treated i.n. with 4 mg/kg, 2 mg/kg, or 1 mg/kg of WT siRNA (WT200, WT100, or WT50, respectively), MM siRNA (MM200, MM100, or MM50, respectively), or PBS for 12h prior to RSV/A2 infection (10^6 PFU). Lungs were harvested at day 4 pi and virus titers determined by immunostaining plaque assay with anti-F protein monoclonal antibody (clone 131-2A) as previously described (64). Data is presented as mean log10 PFU/g titer+SE from n=5 mice/treatment/time-point in 3 separate experiments. The limit of virus detection is between 5-10 PFU/g lung tissue. B) To evaluate the kinetics of siRNA efficacy, mice were intranasally treated with 2 mg/kg WT or MM siRNA or PBS vehicle, and 12h post-treatment, intranasally infected with 106 PFU RSV/A2 (Figure 3.1B). Lungs were collected at days 2, 4, 6, or 8 pi and assayed by immunostaining plaque assay using Vero cells as previously described (64). C) Lung histological pathogenesis was evaluated in WT siRNA-treated (Figure Ci), PBS-treated (Figure Cii), and MM siRNA-treated (Figure Ciii) mice at day 6 pi as previously described (26). Asterisks indicate a significant ($p<0.01$) difference from the control.

(Figure 3.1Ciii) mice. The MM siRNA and PPBS treated mice showed increased peribronchiolar, perivascular, and interstitial lymphocytic infiltrates typical of RSV-mediated pathogenesis (26). These results suggest that the extent of lung pathogenesis following RSV infection appears associated with the virus load.

Bronchoalveolar leukocyte (BAL) response in siRNA-treated mice.

To determine if the reduction in virus titer associated with WT siRNA treatment affected the primary immune response, mice were prophylactically treated with 100 nM (2 mg/kg) WT or MM siRNA or treated with PBS vehicle. Twelve hours post-treatment, the mice were i.n. infected with 10^6 PFU of RSV/A2 and the BAL and sera collected at days 0, 2, 4, 6 or 8 pi. The magnitude of the primary bronchoalveolar leukocyte (BAL) response to RSV infection was highest for mice treated with MM siRNA or PBS compared to WT siRNA-treated mice (Table 3.1). Consistent with peak virus titers (Figure 3.1B), the highest number of BAL cells infiltrating the lung occurred at day 6 pi for all treated mice, and at day 8 pi, all treated mice had similar BAL cell numbers indicating equilibration of BAL cell numbers among the groups of mice.

The BAL cell types were determined during the primary immune response to RSV infection of WT and MM siRNA and PBS treated mice (Table 3.2). Peak $CD4^+$ and $CD8^+$ T cell numbers were detected at day 8 pi with no significant differences ($p<0.0.5$) in total cell numbers between siRNA or PBS-treated mice. A minimal $B220^+$ cell response was observed at all time-points for all treatments. Interestingly, higher numbers of $DX5^+$ cells occurred at day 6 pi in WT or MM siRNA treated mice compared to PBS-treated mice, and MM siRNA treated mice had significantly ($p<0.05$) higher $DX5^+$ cell numbers at day 8 pi compared to WT siRNA or PBS treated mice. This data suggests that siRNA treatment affects the $DX5^+$ cell response at late time-points. It is likely that DX5+ cells are being recruited by cytokines or chemokines produced

Table 3.1 Total bronchoalveolar lavage (BAL) cell numbers during primary RSV infection.

	Day 0	**Day 2**	**Day 4**	**Day 6**	**Day 8**
PBS	2.9×10^4 ($\pm$0.5)	6.0×10^5 $\pm$1.5)$^{\#}$	6.5×10^5 $\pm$1.5)$^{\#}$	1.2×10^6 $\pm$3.1)$^{\#}$	7.0×10^5 +1.2)$^{\#}$
MM	3.1×10^4 ($\pm$0.8)	5.8×10^5 $\pm$2.1)$^{\#}$	7.9×10^5 $\pm$2.8)$^{\#}$	1.4×10^6 $\pm$3.5)$^{\#}$	8.0×10^5 $\pm$1.5)$^{\#}$
WT	3.0×10^4 ($\pm$0.5)	3.9×10^5 $\pm$0.5) $^{*\#}$	3.8×10^5 $\pm$0.2)$^{*\#}$	0.5×10^6 $\pm$3.5) $^{*\#}$	6.5×10^5 $\pm$1.1)$^{\#}$

Note: Mice were intranasally treated with phosphate buffered saline (PBS), 100 nM (2 mg/kg) mismatched siRNA (MM), or 100 nM (2 mg/kg) wild type siRNA (WT) specific for the RSV P gene prior to RSV/A2 infection (10^6 PFU). The total cell numbers ($\pm$standard error) were determined from 3-5 mice/treatment in three separate experiments. * = statistically different ($p<0.05$) from MM and PBS-treated mice; # = statistically different ($p<0.05$) from day 0 response.

Table 3.2. Total number of cell types in the BAL during the primary response to RSV infection.

Cell type and day p.i.	Total no. of cells(SE)		
	WT	**MM**	**PBS**
CD4+			
2	1.5 x 10^3(±3.0)	3.1 x 10^3(±2.2)	2.5 x 10^3(±1.5)
4	16.5 x 10^3(±3.1)	18.5 x 10^3(±2.5)	14.4 x 10^3(±2.8)
6	16.2 x 10^3(±2.1)	16.0 x 10^3(±2.4)	12.2 x 10^3(±1.6)
8	33.5 x 10^3(±5.8)	29.5 x 10^3(±6.0)	31.0 x 10^3(±5.2)
CD8+			
2	2.8 x 10^3(±1.1)	5.8 x 10^3(±2.2)	5.1 x 10^3(±1.5)
4	18.5 x 10^3(±1.8)	17.9 x 10^3(±2.8)	18.9 x 10^3(±2.8)
6	18.4 x 10^3(±2.5)	21.0 x 10^3(±2.1)	19.4 x 10^3(±2.6)
8	48.6 x 10^3(±5.5)	39.5 x 10^3(±5.7)	48.0 x 10^3(±5.5)
B220+			
2	0.09 x 10^3(±0.1)	0.1 x 10^3(±0.1)	0.1 x 10^3(±0.1)
4	0.5 x 10^3(±0.1)	0.2 x 10^3(±0.1)	0.8 x 10^3(±0.1)
6	0.5 x 10^3(±0.5)	0.6 x 10^3(±0.2)	0.5 x 10^3(±0.1)
8	0.3 x 10^3(±0.1)	0.1 x 10^3(±0.1)	0.6 x 10^3(±0.2)
DX5+			
2	2.5 x 10^3(±0.5)†	5.0 x 10^3(±1.1)	4.8 x 10^3(±1.2)
4	5.0 x 10^3(±0.5)	8.0 x 10^3(±1.2)	6.5 x 10^3(±1.3)
6	7.5 x 10^3(±1.2)$^{\dagger\#}$	14.4 x 10^3(±2.8)$^{\#}$	1.3 x 10^3(±1.1)†
8	1.9 x 10^3(±1.1)†	7.0 x 10^3(±1.2)	0.5 x 10^3(±0.5)†
RB6-8C5+			
2	3.8 x 10^3(±0.8)$^{\dagger\#}$	11.8 x 10^3(±1.8)*	10.0 x 10^3(±1.5)*
4	1.9 x 10^3(±0.8)	2.5 x 10^3(±1.8)	3.3 x 10^3(±2.5)
6	5.8 x 10^3(±2.4)	6.0 x 10^3(±1.8)	7.4 x 10^3(±2.0)
8	8.8 x 10^3(±1.4)	8.0 x 10^3(±2.7)	9.8 x 10^3(±0.4)
CD11b+			
2	3.1 x 10^3(±0.5)$^{\dagger\#}$	5.9 x 10^3(±0.5)	5.1 x 10^3(±0.5)
4	10.0 x 10^3(±2.8)	10.8 x 10^3(±2.8)	11.0 x 10^3(±2.2)
6	5.0 x 10^3(±1.2)	4.1 x 10^3(±1.5)	5.9 x 10^3(±1.5)
8	5.5 x 10^3(±1.8)†	9.0 x 10^3(±1.1)	7.5 x 10^3(±1.5)

Note: Mice were intranasally treated with 100 nM (2 mg/kg) wild type (WT) siRNA specific for the RSV P gene, 100 nM (2 mg/kg) mismatched (MM) siRNA, or phosphate buffered saline (PBS) prior to RSV/A2 infection (10^6 PFU). The total cell number ±standard error were determined from 4-5 mice/treatment in three separate experiments using appropriate monoclonal antibodies and flow cytometry. * = statistically different ($p<0.05$) from WT-treated mice; † = statistically different ($p<0.05$) from MM-treated mice; # = statistically different ($p<0.05$) from PBS-treated mice.

in the lung microenvironment at these time-points; however, the mechanism is not known. It may be possible that off-target siRNA activities contribute to these findings, although similar experiments have shown that treatment with similar siRNAs alone do not induce detectable type I IFN levels (6). It is also unlikely that the $DX5^+$ cells are responding directly to the siRNAs because they are rapidly degraded in vivo (38). Since this effect occurs late in the response, i.e. day 6 pi, and only $DX5^+$ cells appear affected by siRNA treatment, there is no evidence that siRNA treatment has a general proinflammatory effect in the lung. A higher number of RB6-$8C5^+$ cells, confirmed by H&E staining as PMN, and $CD11b^+$ cells were detected at day 2 pi in PBS- and MM siRNA-treated mice compared to WT siRNA-treated mice, but no other substantial differences were observed at the other time-points examined. The differences in the recruitment of these innate immune cells between groups of treated mice are unclear, but it is possible that this may be linked to a reduced host cell response to infection associated with a decreased virus load in WT-treated mice (Figure 3.1B).

T cell activation and intracellular cytokine expression.

To determine the level of T cell activation in the BAL, the levels of $CD44^{hi}$ and $CD62L^{lo}$ expression by $CD4^+$ and $CD8^+$ T cells was determined at time-points post-RSV infection (Figure 2A-C). $CD4^+$ and $CD8^+$ T cells from the BAL of mice prophylactically treated with WT or MM siRNA or PBS for 12h prior to infection, i.e. day 0, had very low levels of $CD44^{hi}$ or $CD62L^{lo}$ expression, e.g. the expression levels were below those observed for RSV infected mice at day 2 pi (Figure 3.2A-C). Both $CD4^+$ and $CD8^+$ T cells had increased $CD44^{hi}$ and $CD62L^{lo}$ expression at day 4 pi, and peak numbers of activated $CD4^+$ and $CD8^+$ $CD44^{hi}$ and $CD62L^{lo}$ T cells occurred between days 4 - 6 pi for MM siRNA treated mice (Figure 3.2B), and at days 8 pi for PBS (Figure 3.2A) and WT siRNA treated mice (Figure 3.2C). MM siRNA and PBS treated

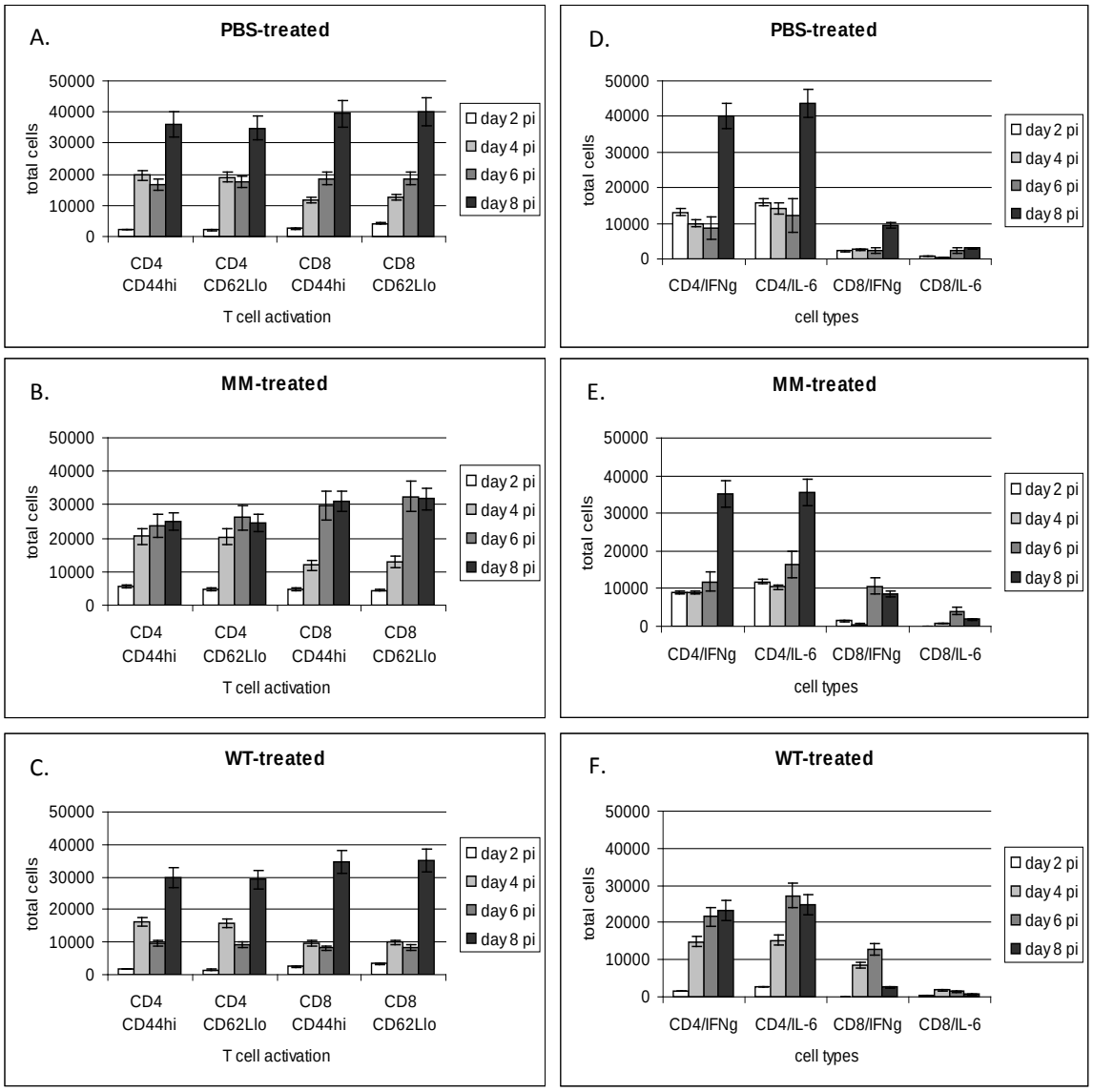


Figure 3.2 CD44^{hi} and CD62L^{lo} expression by CD4^{+} and CD8^{+} T cells in the BAL. Levels of CD44^{hi} and CD62L^{lo} expression by CD4^{+} and CD8^{+} T cells was determined at days 2 – 8 post-RSV infection of PBS treated (A), MM siRNA treated (B) and WT siRNA treated (C) mice. To address the association between CD44^{hi} and CD62L^{lo} T cell expression and Th1/Th2 cytokine expression, the levels of intracellular IFNγ (Th1-type cytokine) and IL-6 (Th2-type cytokine) was determined for CD4^{+} and CD8^{+} T cells at days 2 – 8 pi of PBS treated (D), MM siRNA treated (E) and WT siRNA treated (F) mice. Data is presented as mean total cells±SE from n= 5 mice/treatment/time-point in 3 separate experiments.

mice had significantly ($p<0.05$) higher numbers of activated $CD4^+$ T cells between days 4 - 6 pi compared to WT siRNA-treated mice, and significantly ($p<0.05$) higher numbers of activated $CD8^+$ T cells at 6 pi compared to WT siRNA-treated mice, suggesting that increased T cell activation may be linked to a higher virus load (Figure 3.1B).

To determine the pattern of cytokines expressed by T cells in the BAL, intracellular IFNγ (Th1-type) and IL-6 (Th2-type) expression was determined for $CD4^+$ and $CD8^+$ T cells at days 0 – 8 pi (Figure 2D-F). No significant cytokine response or difference ($p<0.05$) in the total number of $CD4^+$ or $CD8^+$ T cells expressing IFNγ or IL-6 was observed at day 0 (prior to infection). $CD4^+$ T cells in the BAL of any treatment group expressed significantly ($p<0.01$) higher levels of IFNγ and IL-6 at day 8 pi compared to $CD8^+$ T cells, and at other time-points, also expressed higher IFNγ and IL-6 levels than $CD8^+$ T cells. It is interesting to note that the magnitude of IFNγ and IL-6 expression by $CD4^+$ T cells from WT siRNA treated mice was significantly ($p<0.05$) higher at days 4 – 6 pi compared to MM siRNA or PBS-treated mice. Overall, the pattern of $CD4^+$ T cell cytokine expression suggested a mixed Th1/Th2-type cytokine response. However, the pattern of $CD8^+$ T cell cytokine expression in all treated groups, although of lower magnitude compared to $CD4^+$ T cells, was suggestive of a Th1-type response. $CD8^+$ T cell IFNγ levels were significantly ($p<0.05$) higher than IL-6 at day 8 pi in PBS-treated mice, higher at days 6 – 8 pi in MM siRNA-treated mice, and higher at days 4-6 pi in WT siRNA-treated mice (Figure 3.2D-F). These results suggest that the pattern and magnitude of IFNγ and IL-6 expression is not directly linked to the level T cell activation (Figure 3.2A-C), and so not likely to be linked to the virus load.

BAL cell response, virus clearance, and lung pathology during the memory response.

To determine how a reduction in virus titer associated with prophylactic WT siRNA treatment affects the memory response to challenge, mice prophylactically treated with 100 nM (2 mg/kg) WT or MM siRNA or treated with PBS and previously infected with RSV were i.n. challenged 3 weeks later with 10^6 PFU of RSV/A Long and the BAL, lungs, and sera collected at days 0, 2, 4, 6, 8 or 10 pi. BAL cell numbers were similar among the treated mice except at day 6 pi where WT siRNA-treated mice had significantly ($p<0.05$) higher numbers of total BAL cells (Table 3.3). As expected, the total number of BAL cells for any treatment group rapidly increased over the unchallenged but RSV-immune mice.

The total number of $CD4^+$ or $CD8^+$ T cells expressing $CD44^{hi}$ or $CD62L^{lo}$ was determined in the BAL of mice treated with PBS, WT or MM siRNA at days 0 - 10 pi. No significant difference ($p<0.05$) in the total numbers of $CD4^+$ or $CD8^+$ $CD44^{hi}$ and $CD62L^{lo}$ T cells were determined between the treated mice at days 0 - 2 pi (range 500 – 5,500). However, between days 4 – 6 pi in WT siRNA treated mice, the total number of $CD8^+$ $CD44^{hi}$ T cells in the BAL (range 11,000 – 39,500) was significantly ($p<0.05$) higher compared to MM siRNA or PBS treated mice (range 3,200 – 6,500), and $CD8^+$ $CD44^{hi}$ T cell numbers peaked at day 6 pi (range 21,500 – 39,500). In contrast, there was no significant difference in the total number of $CD4^+$ $CD44^{hi}$ T cells at any time point between days 0 – 6 pi (range 500 – 5,500) in PBS, WT or MM siRNA treated mice. Likewise, the total number of $CD8^+$ $CD62L^{lo}$ T cells (range 28,000 – 41,000) was significantly ($p<0.05$) higher between days 4 – 6 pi in the BAL of WT siRNA treated mice compared to PBS or MM siRNA treated mice, and $CD8^+$ $CD62L^{lo}$ T cell numbers at day 6 pi (range 29,500 – 39,000). There was no significant difference ($p<0.05$) in the total

Table 3.3. Total bronchoalveolar lavage (BAL) cell numbers following RSV challenge of immune mice.

	Day 0	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**
PBS	0.8×10^4 ($\pm$0.5)	5.5×10^5 ($\pm$1.1)	6.0×10^5 ($\pm$1.4)	7.2×10^5 ($\pm$2.1)	6.2×10^5 (+1.2)	2.1×10^5 (+1.2)
MM	0.5×10^4 ($\pm$0.5)	5.2×10^5 ($\pm$1.2)	6.7×10^5 ($\pm$1.0)	6.9×10^5 ($\pm$2.0)	6.9×10^5 ($\pm$1.1)	2.3×10^5 ($\pm$1.5)
WT	0.4×10^4 ($\pm$0.5)	5.2×10^5 ($\pm$1.2)	5.8×10^5 ($\pm$1.2)	9.6×10^5 ($\pm$0.8) *#	7.4×10^5 ($\pm$1.3)	2.0×10^5 ($\pm$1.0)

Note: Mice prophylactically treated with WT or MM siRNA or PBS and i.n. infected with 10^6 PFU RSV/A2 were rested for 3 weeks pi then i.n. challenged with 10^6 PFU RSV/Long. BAL was collected at the days indicated post-challenge. The total cell numbers ($\pm$standard error) were determined from 4-5 mice/treatment in three separate experiments. * = statistically different ($p<0.05$) from MM-treated mice; # = statistically different ($p<0.05$) from PBS-treated mice.

number of $CD4^+$ $CD62L^{lo}$ T cells at any time point between days 0 – 6 pi (range 1000 – 5,500) in PBS, WT or MM siRNA treated mice. Between days 8 – 10 pi, the total numbers of $CD8^+CD44^{hi}$ or $CD62L^{lo}$ T cells in the BAL of WT siRNA treated were similar (range 20,500 – 27,500), and remained significantly higher ($p<0.05$) compared to $CD4^+CD44^{hi}$ or $CD62L^{lo}$ T cells in the BAL of MM siRNA or PBS treated mice (range 2,200 – 4,500). These results suggest that $CD8^+CD44^{hi}$ and $CD8^+CD62L^{lo}$ are a principal memory T cell population in the RSV memory response in WT siRNA treated mice, and that memory $CD8^+$ T cells are more predominant than $CD4^+$ T cells in the BAL.

In addition to investigating the T cell types, the numbers of innate immune cell types in the BAL was determined. No significant difference ($p<0.05$) in the total number of $DX5^+$ or RB6-$8C5^+$ cells was detected at any time point examined in PBS, MM or WT siRNA treated mice. However, there were higher numbers of $DX5^+$ cells (range 17,000-24,000) in WT siRNA treated mice between days 4 - 6 pi compared to PBS or MM siRNA treated mice (range 14,000 – 21,500), and the total number of RB6-$8C5^+$ cells were higher in the BAL of WT siRNA treated mice at days 4 - 6 pi (range 16,000 – 22,000) compared to PBS- or MM siRNA-treated mice (range 13,500 – 16,000).

The lung virus titers in the treated mice were determined. The highest lung virus titers measured occurred at day 2 post-challenge in all treated groups; however, virus titers were significantly lower ($p<0.05$) at day 4 post-challenge in WT siRNA treated mice compared to MM siRNA or PBS treated mice (Figure 3.3A). No virus was detectable in the lungs of WT siRNA treated mice at day 4 post-challenge, however similar levels of virus (1.8 – 2.6 $\log_{10}$ pfu g/lung tissue) was detected in the lungs of MM siRNA and PBS treated mice. All treated groups of mice cleared virus by day 6 pi. No lung histopathology was evident at day 0 prior to challenge

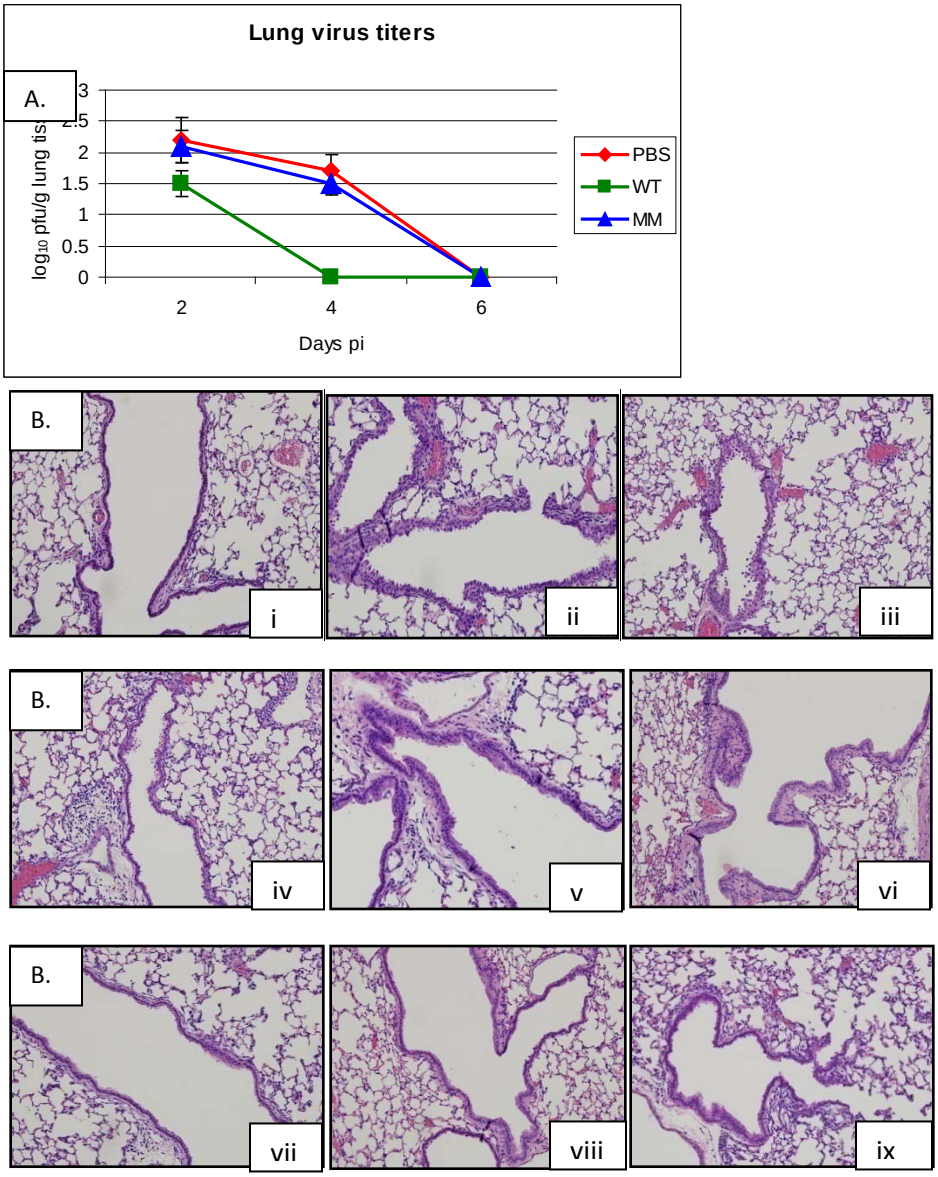


Figure 3.3 Lung virus titers in the memory response to RSV challenge. To determine the effect of treatment on lung virus titers in the memory response to RSV challenge, mice prophylactically treated with WT siRNA (WT), MM siRNA (MM) or PBS were challenged with 10^6 PFU RSV/A Long (A). The lungs were harvested at days 2, 4, and 6 post-challenge to determine virus titers as previously described (64). Data is presented as mean $\log_{10}$ pfu/g titer$\pm$SE from n=5 mice/treatment/time-point in 3 separate experiments. Lung histopathology was evaluated in the treated mice challenged with RSV (B). No lung histopathology was evident at day 0 prior to challenge for any treated group (data not shown). Histopathology was evaluated for WT siRNA treated at days 2, 4 and 6 pi (Figure 3Bi, Biv, Bvi, respectively), for PBS-treated mice at days 2, 4 or 6 pi (Figure 3Bii, Bv and 3Bvii, respectively), and for MM siRNA treated mice at days 2, 4 or 6 pi (Figure 3Biii, Bvi, and Bix, respectively) as previously described (26). Asterisks indicate a significant ($p<0.01$) difference from the control.

for any treated group, and no substantial histopathology was detected in WT siRNA treated at days 2, 4 or 6 pi (Figure 3.3Bi, Biv, Bvi, respectively). However, PBS and MM siRNA treated mice showed similar levels of peribronchiolar, perivascular, and interstitial lymphocytic infiltrates typical of RSV-mediated pathogenesis (26) at day 2 pi (Figure 3.3Bii and 3.3Biii, respectively), day 4 pi (Figure 3.3Bv and 3Bvi, respectively), and 6 pi (Figure 3.3Bvii and 3.3Bix, respectively). These results show that prophylactic WT siRNA treatment, despite decreasing the virus load during the primary immune response, sufficiently primes for a robust memory immune response to RSV challenge that is not linked with substantial pulmonary disease pathogenesis compared to MM siRNA or PBS treated mice.

Anti-RSV antibody responses.

The evidence that mice prophylactically treated with WT siRNA recovered from virus challenge faster (days 2-4 pi) compared to MM siRNA- or PBS-treated mice (days 4-6 pi; Figure 3A) suggested a difference in the quality of the memory response related to treatment. To determine if the differences were in part related to the antibody response, the IgG1 (Th2-type) and IgG2a (Th1-type) anti-RSV antibody responses were examined in sera collected from the treated mice (Figure 3.4). The antibody response was Th1/Th2 mixed with no significant differences in IgG1 or IgG2a responses between the treated groups of mice. WT siRNA-treated mice had slightly higher IgG1 and IgG2a antibody responses at day 10 pi compared to MM siRNA or PBS treated mice, but this difference likely does not explain the more rapid viral clearance by this group. Neutralizing antibody titers were also evaluated in the sera from WT and MM siRNA and PBS treated mice at day 6 pi; however, no statistical differences ($p<0.05$) in efficacy were determined where the neutralizing anti-RSV serum titers ranged from 6.0 to 6.9 reciprocal mean $\log_2$ between the treated mice.

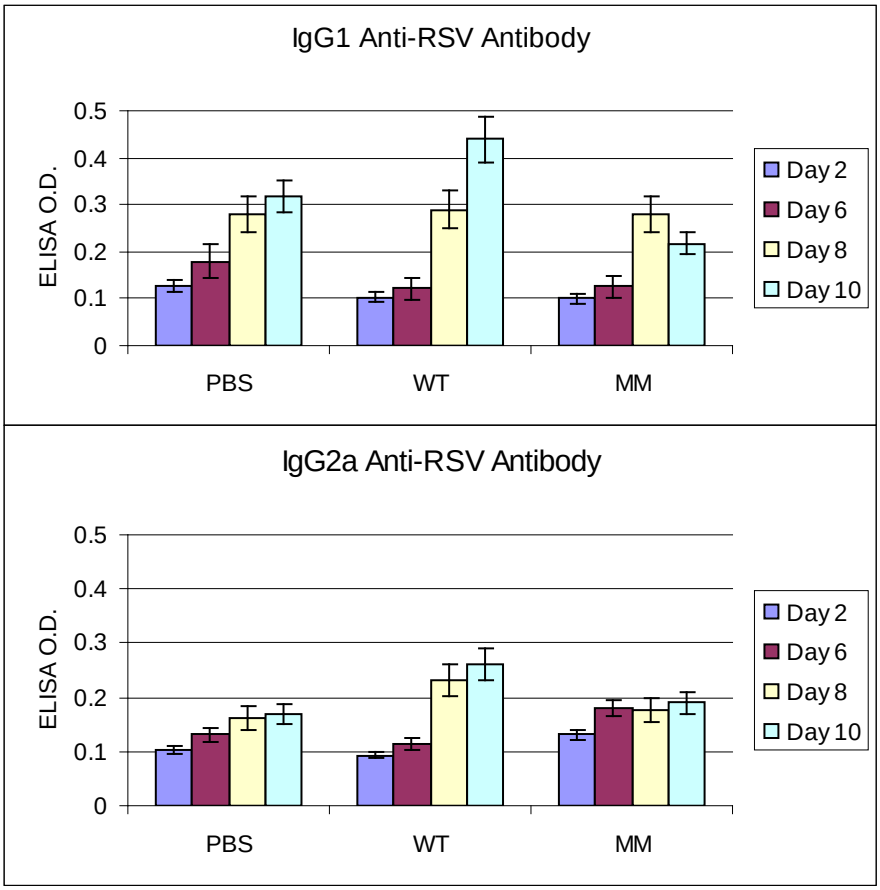


Figure 3.4 RSV-specific antibody responses in treated mice. RSV-specific IgG1 or IgG2a antibody responses in siRNA or PBS treated mice were determined at days 2, 6, 8, and 10 pi in mice challenged with 10^6 PFU RSV/A Long. Data is presented as mean ELISA OD$\pm$SE from n= 5 mice/treatment/time-point in 3 separate experiments.

Memory T cell responses.

The higher number of $CD44^{hi}$ and $CD62L^{lo}$ $CD8^+$ T cells in the BAL of WT siRNA treated memory mice compared to MM siRNA or PBS treated mice indicated that the memory $CD8^+$ T cell response may be associated with enhanced virus clearance. To address this possibility, the memory $CD8^+$ T cell response to the immunodominant RSV M2 peptide ($M2_{82–90}$)(36) was determined at days 0 – 6 pi using M2-K^d tetramer staining (Figure 3.5). The results show that significantly ($p<0.05$) higher total numbers of M2-specific $CD8^+$+ T cells trafficked to the lung between days 4 - 6 pi in WT siRNA-treated mice compared to MM siRNA or PBS treated mice (Figure 3.5A), a finding consistent with the results showing faster virus clearance in these treated mice compared to MM siRNA- or PBS-treated mice (Figure 3.3A). Notably, there was a significantly ($p<0.01$) higher total number of M2-specific $CD8^+$ T cells expressing intracellular IFNγ between days 4-6 pi in WT siRNA-treated mice compared to MM siRNA or PBS treated mice (Figure 3.5B). These data suggest that $CD8^+$ memory T cell response in WT siRNA-treated mice likely contribute to the enhanced rate of virus clearance.

To determine the pattern of cytokines expressed by memory T cells in the BAL, intracellular IFNγ (Th1-type) and IL-6 (Th2-type) expression were determined for $CD4^+$ and $CD8^+$ T cells at days 0 – 6 pi. The total number of $CD4^+$ T cells expressing IFNγ was similar between days 4 – 6 pi for MM siRNA or PBS treated mice (range 20,000 – 27,500). In contrast, the total number of $CD4^+$ T cells expressing IFNγ from WT siRNA-treated mice was significantly ($p<0.05$) higher (range 38,500 – 42,500). No significant difference ($p<0.05$) in the total number of $CD4^+$ T cells expressing IL-6 was observed at any time-point among the groups of treated mice (range 3,500 – 10,500). A similar trend for IFNγ and IL-6 expression was observed for $CD8^+$ T cells from MM siRNA- or PBS-treated mice where the total number of

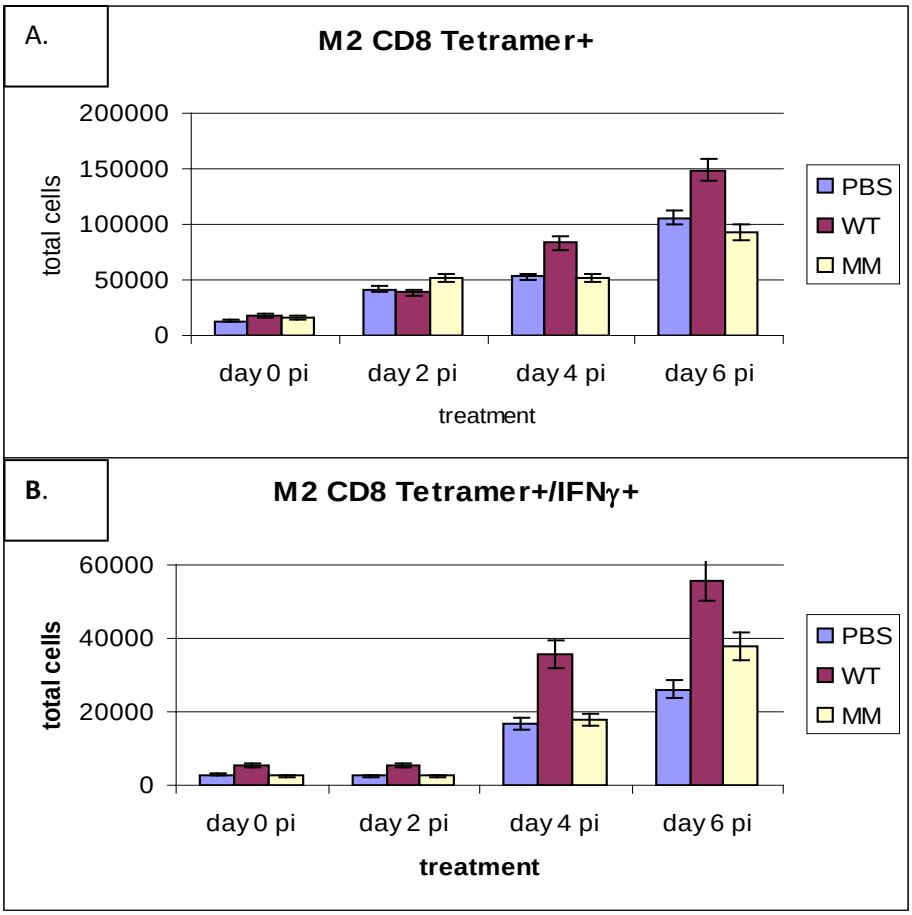


Figure 3.5 $CD8^+$ T cell memory response to the RSV M2 peptide. The $CD8^+$ T cell memory response to the immunodominant RSV M2 $peptide_{82–90}$ (36) was determined at days 0 – 6 pi using M2-K^d tetramer staining. The total number of M2 $tetramer^+$ cells was determined at days 0, 2, 4 and 6 pi (A). Data is presented as mean total cells±SE from n= 5 mice/treatment/time-point in 3 separate experiments. The total number of M2 $tetramer^+$ cells expressing intracellular IFNγ was determined at days 0, 2, 4 and 6 pi (B). Data is presented as mean total cells±SE from n= 5 mice/treatment/time-point in 3 separate experiments.

$CD8^+$ T cells expressing IFNγ between days 4 – 6 pi ranged from 28,000 – 40,000, and in contrast, the total number of $CD8^+$ T cells expressing IFNγ from WT siRNA-treated mice was significantly ($p<0.01$) higher (range 59,000 – 72,000). No substantial difference in IL-6 expression was observed (range 2,500 – 4,500) among the groups of treated mice. The higher total number of $CD8^+$ T cells expressing IFNγ from WT siRNA-treated mice is consistent with the finding of higher total numbers of M2-specific $CD8^+$ T cells in WT siRNA-treated mice compared to MM siRNA or PBS treated mice (Figure 3.5A) - features that may be linked to more rapid virus clearance in memory mice (Figure 3.3A).

Discussion

The most successful approach to control RSV infection to date has been prevention or treatment with anti-RSV antibodies. RSV-immune globulin (RespiGam™) was the forerunner for use in children less than two years of age at high-risk of RSV infection (70). This treatment was basically supplanted with palivizumab, an IgG1 humanized monoclonal antibody that selectively binds the RSV F protein and is neutralizing (27, 70). Palivizumab has been shown to reduce the rate of hospitalization and is the current primary means of RSV prevention as no safe and efficacious vaccine is available (24). Despite the utility of anti-RSV antibodies to control infection, new antiviral drug approaches are being sought that have potentially broader application and efficacy.

The use of RNAi drugs to target viruses as a disease intervention strategy is an approach that continues to grow both in academia and industry as many important human viruses lack efficacious vaccines and anti-viral drugs, an example being RSV. RNAi drugs whose active component are siRNAs appear to be ideal for inhibiting respiratory viruses such as RSV because there are multiple siRNA targets in conserved viral genes, and because siRNAs can be targeted

to sites of infection, i.e. the respiratory epithelia. It is also important to consider that RNAi drug efficacy is independent of immune status, thus these drugs may be an effective treatment strategy in the very young, elderly or immune compromised individuals that are often susceptible to severe disease particularly following RSV infection (1, 23, 34).

RNAi of RSV replication was demonstrated using a 21-nucleotide long double-stranded interfering RNA targeting the RSV P gene (6). Using this approach it was previously shown that RSV silencing by RNAi is highly efficient in that nanomolar siRNA concentrations led to RSV silencing both in vitro and in vivo (4, 6), and the effect was highly specific in that siRNAs targeted only the gene of interest and silencing was not related to induction of an IFN response. Although the efficacy of siRNA treatment to reduce virus replication was clear, it remains unclear how siRNA treatment affects the host immune response to infection or subsequent challenge. This issue is not trivial, and is particularly important for RSV, where natural infection does not seem to induce durable immunity and individuals may be repeatedly infected with the same and different strains of RSV (9, 40, 50, 59).

In this study, we examined the effect of prophylactic siRNA treatment targeting the RSV P gene on the primary and memory immune response to RSV infection or challenge. This study addresses questions related to the pathogenic threshold associated with virus load and the quality of the innate and adaptive immune response to RSV infection and challenge. This relationship is important as RSV disease severity is thought to be principally due to the host immune response (19, 46, 66), and the history of attenuated RSV vaccine studies has often revealed inadequate immunogenicity (16, 22, 41, 67), suggesting the virus load is relevant in the outcome of the memory response. To determine if some of the immune responses associated with WT siRNA prophylaxis could be emulated by titering down RSV/A2 during primary infection, we also

performed a series of experiments in which mice were i.n. inoculated with 10^6, 10^5, 10^4 or 10^3 PFU of RSV/A2. The results suggested that the quality of the primary immune response was different at the two time-points examined, i.e. days 4 and 6 pi compared to WT siRNA treated mice, particularly in the total number and type of BAL cells recruited to the lung, as well as in the pattern of intracellular cytokine expression. These results were not unexpected because Toll-like receptors (TLRs) are affected by RSV F and G proteins (21, 28, 37, 53), and decreasing the virus concentration for infection effectively decreases the amount of these viral surface proteins for interaction with TLRs which impacts the induction of innate and adaptive immune responses. Also, mice prophylactically treated with WT siRNA receive a virus inoculum of 10^6 PFU, but the level of virus replication is limited by RNAi-mediated P gene silencing following virus infection – not because of virus titer during infection. Thus, this aspect of the study was confounding and not pursued further. The significance of an appropriate host response to RSV disease severity is evident in formalin-inactivated RSV vaccine studies where disease enhancement has been associated with Th2-type cytokine shifts and remarkable changes in pulmonary cell trafficking, particularly the development of pulmonary eosinophilia (11). These features are linked to quantitative differences in the pattern, type, and magnitude of the host response to RSV and RSV proteins. We show that a reduction in the lung virus load mediated WT siRNA is associated with reduced pulmonary cell infiltration, and largely reduced levels of cell activation, cytokine expression and pathology during the primary response to infection. These findings are consistent with a reduced inflammatory response and the hypothesis that the virus load contributes to disease severity in part due to the host immune response. However, the reduced inflammatory response associated with WT siRNA treatment prophylaxis did not detrimentally affect the memory T cell or antibody response to RSV challenge. Indeed, the

memory T cell response was robust in WT siRNA treated mice. It has been shown that $CD4^+$ and $CD8^+$ T cells make significant independent contributions to the restriction of RSV replication in the mouse model (17, 25, 33, 51, 54), and a dominant role for $CD4^+$ T cells is help for effective $CD8^+$ T cell priming (69). Since the $CD4^+$ T cell response was similar between WT and MM siRNA treated mice during the primary immune response, but the $CD8^+$ T cell memory response was by several parameters more dynamic for WT siRNA treated mice, the results suggest that $CD4^+$ priming of $CD8^+$ T cells is effective at conditions of reduced virus load associated with WT siRNA treatment. In addition, as $CD8^+$ T cells (in particular RSV M2-specific $CD8^+$ T cells) have been shown to have an important role in the regulation of Th2 $CD4^+$ T cell responding to RSV infection as well as regulating lung pathogenesis (25, 33, 48, 54), the results suggest that $CD8^+$ T cells were appropriately primed in WT siRNA treated mice as the memory response was associated with a mixed Th1/Th2-type cytokine and antibody responses and limited lung pathology was observed.

As indicated in the study, one of the features associated with the vigorous RSV-specific memory response in WT siRNA treated mice was the development of high numbers of RSV M2-tetramer specific, IFNγ expressing $CD8^+$ T cells. RSV M2-specific $CD8^+$ T cells have been shown to regulate and reduce Th2-mediated pathology in an IFNγ-independent manner (48), a finding that may be consistent with limited level of lung histopathology observed in WT siRNA treated mice compared to MM siRNA or PBS treated mice. It is not clear what accounts for the differences in RSV M2-tetramer specific $CD8^+$ T cell numbers between treated mice; however, it is possible that the pattern of IL-6 expression may affect the regulation of this response. IL-6 was expressed earlier and to generally higher levels in WT siRNA treated mice compared to MM siRNA or PBS treated mice. IL-6 has been shown to have an important role in the development

of T cell memory to influenza virus, and specifically that its ability to potentially suppress $CD4^+CD25^+$ regulatory T cells (39).

The implications of this study are that RNAi drugs can be developed that target conserved RSV genes, e.g. P gene, and can be used in vivo to reduce virus replication and diminish parameters of disease pathogenesis without impairing priming of the memory response. The findings of this study suggest that virus load may be linked with disease pathogenesis in part due to the magnitude of the host immune response. However, pathogenesis does not appear to be directly linked to the RSV-specific $CD8^+$ T cell response because WT siRNA treated mice have higher numbers of RSV M2-specific $CD8^+$ T cells and have low levels of pulmonary pathogenesis compared to MM siRNA or PBS treated mice. It is possible that the reduced virus load related to WT siRNA treatment limits the threshold required for some RSV genes to modify aspects of RSV immunity leading to a more appropriate anti-viral response. For example, RSV G protein expression has been linked to modified cytokine and chemokine response by BAL cells, altered trafficking and responses by $CX3CR1^+$ T cells, and molecular mimicry of fractalkine-mediated responses (31, 41, 59, 63, 64, 66). The findings from these studies provide a foundation for new RSV disease intervention studies that employ RNAi technologies, and may offer a new direction in treatments for RSV.

Acknowledgements

We thank Jackelyn Crabtree, Jamie Barber, and Les Jones for their assistance in maintaining cell cultures, flow cytometry, and discussions, and support from the Georgia Research Alliance.

References

1. 2007. Brief report: respiratory syncytial virus activity--United States, July 2006-November 2007. MMWR Morb Mortal Wkly Rep **56:**1263-5.

2. **Barik, S.** 2004. Control of nonsegmented negative-strand RNA virus replication by siRNA. Virus Res **102:**27-35.

3. **Barik, S.** 2004. Development of gene-specific double-stranded RNA drugs. Ann Med **36:**540-51.

4. **Barik, S., and V. Bitko.** 2006. Prospects of RNA interference therapy in respiratory viral diseases: update 2006. Expert Opin Biol Ther **6:**1151-60.

5. **Becker, Y.** 2006. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy--a review. Virus Genes **33:**235-52.

6. **Bitko, V., A. Musiyenko, O. Shulyayeva, and S. Barik.** 2005. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med **11:**50-5.

7. **Black, C. P.** 2003. Systematic review of the biology and medical management of respiratory syncytial virus infection. Respir Care **48:**209-31; discussion 231-3.

8. **Braciale, T. J.** 2005. Respiratory syncytial virus and T cells: interplay between the virus and the host adaptive immune system. Proc Am Thorac Soc **2:**141-6.

9. **Broor, S., S. Parveen, P. Bharaj, V. S. Prasad, K. N. Srinivasulu, K. M. Sumanth, S. K. Kapoor, K. Fowler, and W. M. Sullender.** 2007. A prospective three-year cohort study of the epidemiology and virology of acute respiratory infections of children in rural India. PLoS ONE **2:**e491.

10. **Cane, P. A., and C. R. Pringle.** 1995. Molecular epidemiology of respiratory syncytial virus: a review of the use of reverse transcription-polymerase chain reaction in the analysis of genetic variability. Electrophoresis **16:**329-33.

11. **Castilow, E. M., M. R. Olson, and S. M. Varga.** 2007. Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. Immunol Res **39:**225-39.

12. **Chang, J., and T. J. Braciale.** 2002. Respiratory syncytial virus infection suppresses lung CD8+ T-cell effector activity and peripheral CD8+ T-cell memory in the respiratory tract. Nat Med **8:**54-60.

13. **Chang, J., A. Srikiatkhachorn, and T. J. Braciale.** 2001. Visualization and characterization of respiratory syncytial virus F-specific CD8(+) T cells during experimental virus infection. J Immunol **167:**4254-60.

14. **Chavez-Bueno, S., A. Mejias, R. A. Merryman, N. Ahmad, H. S. Jafri, and O. Ramilo.** 2007. Intravenous palivizumab and ribavirin combination for respiratory syncytial virus disease in high-risk pediatric patients. Pediatr Infect Dis J **26:**1089-93.

15. **Connors, M., A. B. Kulkarni, P. L. Collins, C. Y. Firestone, K. L. Holmes, H. C. Morse, 3rd, and B. R. Murphy.** 1992. Resistance to respiratory syncytial virus (RSV) challenge induced by infection with a vaccinia virus recombinant expressing the RSV M2 protein (Vac-M2) is mediated by CD8+ T cells, while that induced by Vac-F or Vac-G recombinants is mediated by antibodies. J Virol **66:**1277-81.

16. **Crowe, J. E., Jr.** 1998. Immune responses of infants to infection with respiratory viruses and live attenuated respiratory virus candidate vaccines. Vaccine **16:**1423-32.

17. **Crowe, J. E., Jr., C. Y. Firestone, and B. R. Murphy.** 2001. Passively acquired antibodies suppress humoral but not cell-mediated immunity in mice immunized with live attenuated respiratory syncytial virus vaccines. J Immunol **167:**3910-8.

18. **Dakhama, A., Y. M. Lee, and E. W. Gelfand.** 2005. Virus-induced airway dysfunction: pathogenesis and biomechanisms. Pediatr Infect Dis J **24:**S159-69, discussion S166-7.

19. **DeVincenzo, J. P.** 2005. Factors predicting childhood respiratory syncytial virus severity: what they indicate about pathogenesis. Pediatr Infect Dis J **24:**S177-83, discussion S182.

20. **Ebbert, J. O., and A. H. Limper.** 2005. Respiratory syncytial virus pneumonitis in immunocompromised adults: clinical features and outcome. Respiration **72:**263-9.

21. **Ehl, S., R. Bischoff, T. Ostler, S. Vallbracht, J. Schulte-Monting, A. Poltorak, and M. Freudenberg.** 2004. The role of Toll-like receptor 4 versus interleukin-12 in immunity to respiratory syncytial virus. Eur J Immunol **34:**1146-53.

22. **Englund, J. A.** 1999. Prevention strategies for respiratory syncytial virus: passive and active immunization. J Pediatr **135:**38-44.

23. **Falsey, A. R.** 2007. Respiratory syncytial virus infection in adults. Semin Respir Crit Care Med **28:**171-81.

24. **Feltes, T. F., and H. M. Sondheimer.** 2007. Palivizumab and the prevention of respiratory syncytial virus illness in pediatric patients with congenital heart disease. Expert Opin Biol Ther **7:**1471-80.

25. **Graham, B. S., L. A. Bunton, P. F. Wright, and D. T. Karzon.** 1991. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest **88:**1026-33.

26. **Graham, B. S., M. D. Perkins, P. F. Wright, and D. T. Karzon.** 1988. Primary respiratory syncytial virus infection in mice. J Med Virol **26:**153-62.

27. **Greenough, A.** 2002. Respiratory syncytial virus infection: clinical features, management, and prophylaxis. Curr Opin Pulm Med **8:**214-7.

28. **Groskreutz, D. J., M. M. Monick, L. S. Powers, T. O. Yarovinsky, D. C. Look, and G. W. Hunninghake.** 2006. Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. J Immunol **176:**1733-40.

29. **Hacking, D., and J. Hull.** 2002. Respiratory syncytial virus--viral biology and the host response. J Infect **45:**18-24.

30. **Hannon, G. J.** 2002. RNA interference. Nature **418:**244-51.

31. **Harcourt, J., R. Alvarez, L. P. Jones, C. Henderson, L. J. Anderson, and R. A. Tripp.** 2006. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. J Immunol **176:**1600-8.

32. **Huang, D. D.** 2008. The potential of RNA interference-based therapies for viral infections. Curr HIV/AIDS Rep **5:**33-9.

33. **Hussell, T., C. J. Baldwin, A. O'Garra, and P. J. Openshaw.** 1997. CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. Eur J Immunol **27:**3341-9.

34. **Khanna, N., A. F. Widmer, M. Decker, I. Steffen, J. Halter, D. Heim, M. Weisser, A. Gratwohl, U. Fluckiger, and H. H. Hirsch.** 2008. Respiratory syncytial virus infection in patients with hematological diseases: single-center study and review of the literature. Clin Infect Dis **46:**402-12.

35. **Kulkarni, A. B., M. Connors, C. Y. Firestone, H. C. Morse, 3rd, and B. R. Murphy.** 1993. The cytolytic activity of pulmonary CD8+ lymphocytes, induced by infection with a vaccinia virus recombinant expressing the M2 protein of respiratory syncytial virus (RSV), correlates with resistance to RSV infection in mice. J Virol **67:**1044-9.

36. **Kulkarni, A. B., H. C. Morse, 3rd, J. R. Bennink, J. W. Yewdell, and B. R. Murphy.** 1993. Immunization of mice with vaccinia virus-M2 recombinant induces epitope-specific and cross-reactive Kd-restricted CD8+ cytotoxic T cells. J Virol **67:**4086-92.

37. **Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson, and R. W. Finberg.** 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol **1:**398-401.

38. **Layzer, J. M., A. P. McCaffrey, A. K. Tanner, Z. Huang, M. A. Kay, and B. A. Sullenger.** 2004. In vivo activity of nuclease-resistant siRNAs. RNA **10:**766-71.

39. **Longhi, M. P., K. Wright, S. N. Lauder, M. A. Nowell, G. W. Jones, A. J. Godkin, S. A. Jones, and A. M. Gallimore.** 2008. Interleukin-6 is crucial for recall of influenza-specific memory CD4 T cells. PLoS Pathog **4:**e1000006.

40. **Madhi, S. A., L. Kuwanda, C. Cutland, and K. P. Klugman.** 2006. Five-year cohort study of hospitalization for respiratory syncytial virus associated lower respiratory tract infection in African children. J Clin Virol **36:**215-21.

41. **Mahalingam, S., J. Schwarze, A. Zaid, M. Nissen, T. Sloots, S. Tauro, J. Storer, R. Alvarez, and R. A. Tripp.** 2006. Perspective on the host response to human metapneumovirus infection: what can we learn from respiratory syncytial virus infections? Microbes Infect **8:**285-93.

42. **Manjunath, N., P. Kumar, S. K. Lee, and P. Shankar.** 2006. Interfering antiviral immunity: application, subversion, hope? Trends Immunol **27:**328-35.

43. **Meister, G., and T. Tuschl.** 2004. Mechanisms of gene silencing by double-stranded RNA. Nature **431:**343-9.

44. **Mejias, A., S. Chavez-Bueno, H. S. Jafri, and O. Ramilo.** 2005. Respiratory syncytial virus infections: old challenges and new opportunities. Pediatr Infect Dis J **24:**S189-96, discussion S196-7.

45. **Mello, C. C., and D. Conte, Jr.** 2004. Revealing the world of RNA interference. Nature **431:**338-42.

46. **Moore, M. L., and R. S. Peebles, Jr.** 2006. Respiratory syncytial virus disease mechanisms implicated by human, animal model, and in vitro data facilitate vaccine strategies and new therapeutics. Pharmacol Ther **112:**405-24.

47. **Murata, Y., and A. R. Falsey.** 2007. Respiratory syncytial virus infection in adults. Antivir Ther **12:**659-70.

48. **Olson, M. R., and S. M. Varga.** 2007. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. J Immunol **179:**5415-24.

49. **Openshaw, P. J.** 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am J Respir Crit Care Med **152:**S59-62.

50. **Parveen, S., S. Broor, S. K. Kapoor, K. Fowler, and W. M. Sullender.** 2006. Genetic diversity among respiratory syncytial viruses that have caused repeated infections in children from rural India. J Med Virol **78:**659-65.

51. **Plotnicky-Gilquin, H., A. Robert, L. Chevalet, J. F. Haeuw, A. Beck, J. Y. Bonnefoy, C. Brandt, C. A. Siegrist, T. N. Nguyen, and U. F. Power.** 2000. CD4(+) T-cell-mediated antiviral protection of the upper respiratory tract in BALB/c mice following

parenteral immunization with a recombinant respiratory syncytial virus G protein fragment. J Virol **74:**3455-63.

52. **Power, U. F.** 2008. Respiratory syncytial virus (RSV) vaccines--two steps back for one leap forward. J Clin Virol **41:**38-44.

53. **Shingai, M., M. Azuma, T. Ebihara, M. Sasai, K. Funami, M. Ayata, H. Ogura, H. Tsutsumi, M. Matsumoto, and T. Seya.** 2008. Soluble G protein of respiratory syncytial virus inhibits Toll-like receptor 3/4-mediated IFN-beta induction. Int Immunol.

54. **Srikiatkhachorn, A., and T. J. Braciale.** 1997. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. J Exp Med **186:**421-32.

55. **Srikiatkhachorn, A., and T. J. Braciale.** 1997. Virus-specific memory and effector T lymphocytes exhibit different cytokine responses to antigens during experimental murine respiratory syncytial virus infection. J Virol **71:**678-85.

56. **Stram, Y., and L. Kuzntzova.** 2006. Inhibition of viruses by RNA interference. Virus Genes **32:**299-306.

57. **Tebbey, P. W., C. A. Scheuer, J. A. Peek, D. Zhu, N. A. LaPierre, B. A. Green, E. D. Phillips, A. R. Ibraghimov, J. H. Eldridge, and G. E. Hancock.** 2000. Effective

mucosal immunization against respiratory syncytial virus using purified F protein and a genetically detoxified cholera holotoxin, CT-E29H. Vaccine **18:**2723-34.

58. **Tijsterman, M., and R. H. Plasterk.** 2004. Dicers at RISC; the mechanism of RNAi. Cell **117:**1-3.

59. **Tripp, R. A.** 2004. Pathogenesis of respiratory syncytial virus infection. Viral Immunol **17:**165-81.

60. **Tripp, R. A., A. Barskey, L. Goss, and L. J. Anderson.** 2002. Substance P receptor expression on lymphocytes is associated with the immune response to respiratory syncytial virus infection. J Neuroimmunol **129:**141-53.

61. **Tripp, R. A., A. Dakhama, L. P. Jones, A. Barskey, E. W. Gelfand, and L. J. Anderson.** 2003. The G glycoprotein of respiratory syncytial virus depresses respiratory rates through the CX3C motif and substance P. J Virol **77:**6580-4.

62. **Tripp, R. A., L. Jones, and L. J. Anderson.** 2000. Respiratory syncytial virus G and/or SH glycoproteins modify CC and CXC chemokine mRNA expression in the BALB/c mouse. J Virol **74:**6227-9.

63. **Tripp, R. A., L. P. Jones, L. M. Haynes, H. Zheng, P. M. Murphy, and L. J. Anderson.** 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol **2:**732-8.

64. **Tripp, R. A., D. Moore, L. Jones, W. Sullender, J. Winter, and L. J. Anderson.** 1999. Respiratory syncytial virus G and/or SH protein alters Th1 cytokines, natural killer cells, and neutrophils responding to pulmonary infection in BALB/c mice. J Virol **73:**7099-107.

65. **Tripp, R. A., D. Moore, J. Winter, and L. J. Anderson.** 2000. Respiratory syncytial virus infection and G and/or SH protein expression contribute to substance P, which mediates inflammation and enhanced pulmonary disease in BALB/c mice. J Virol **74:**1614-22.

66. **Tripp, R. A., C. Oshansky, and R. Alvarez.** 2005. Cytokines and respiratory syncytial virus infection. Proc Am Thorac Soc **2:**147-9.

67. **van Drunen Littel-van den Hurk, S., J. W. Mapletoft, N. Arsic, and J. Kovacs-Nolan.** 2007. Immunopathology of RSV infection: prospects for developing vaccines without this complication. Rev Med Virol **17:**5-34.

68. **Welliver, R. C.** 2003. Respiratory syncytial virus and other respiratory viruses. Pediatr Infect Dis J **22:**S6-10; discussion S10-2.

69. **Williams, M. A., B. J. Holmes, J. C. Sun, and M. J. Bevan.** 2006. Developing and maintaining protective CD8+ memory T cells. Immunol Rev **211:**146-53.

70. **Wu, H., D. S. Pfarr, G. A. Losonsky, and P. A. Kiener.** 2008. Immunoprophylaxis of RSV infection: advancing from RSV-IGIV to palivizumab and motavizumab. Curr Top Microbiol Immunol **317:**103-23.

71. **Zhang, L., M. E. Peeples, R. C. Boucher, P. L. Collins, and R. J. Pickles.** 2002. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J Virol **76:**5654-66.

72. **Zhao, D. C., T. Yan, L. Li, S. You, and C. Zhang.** 2007. Respiratory syncytial virus inhibits interferon-alpha-inducible signaling in macrophage-like U937 cells. J Infect **54:**393-8.

CHAPTER 4

ANTIBODIES REACTIVE TO THE CENTRAL CONSERVED REGION OF THE RESPIRATORY SYNCYTIAL VIRUS (RSV) G PROTEIN BLOCK G PROTEIN BINDING TO THE FRACTALKINE RECEPTOR, CX3CR1, AND REDUCE DISEASE PATHOGENESIS IN MICE

Wenliang Zhang*, Les P. Jones*, Lia M. Haynes#, Jennifer L. Harcourt#, Larry J. Anderson# and Ralph A. Tripp* . To be submitted to Journal of Virology

Abstract

Respiratory syncytial virus (RSV) is a major cause of morbidity and some mortality in infants, young children and the elderly worldwide. Currently there is no safe and effective vaccine. The anti-viral drugs to control RSV infection are limited. RSV has been shown to modulate the host immune response to infection by various mechanisms. RSV G protein contains CX3C chemokine motif that has been shown to bind to the fractalkine receptor, CX3CR1, and modulate the immune responses by CX3CR1+ cells. Evidence in mice suggests that G protein CX3C-CX3CR1 interaction contributes to immune evasion and may contribute to disease pathogenesis. This study determined if antibodies reactive to epitopes across the central conserved region of G protein blocked G protein interaction with CX3CR1. Using an *in vitro* binding model of human 293 cells transfected with CX3CR1, antisera derived from mice vaccinated with G protein peptides or polypeptides were tested *in vitro* for their ability to prevent purified RSV G protein from binding to CX3CR1 by flow cytometry. The results show that antibodies specific for the central conserved region of the G protein have higher blocking activities than the ones specific for N- and C- terminal regions. *In vivo*, we have also shown that antibodies specific for the central conserved region can reduce mouse body weight loss, significantly reduce lung pathology and decrease lung virus titers. These results suggest that vaccines can be made which induce G protein CX3C-CX3CR1 blocking antibodies, and that this vaccine strategy may be useful to prevent G protein immune modulation and disease pathogenesis.

Introduction

Human respiratory syncytial virus (RSV) is one of the most important human pathogens that cause serious lower respiratory tract diseases in infants, young children, the elderly, and the

immune comprised worldwide (4, 8, 17, 18). In the US, RSV is estimated to be associated with more than 120,000 respiratory hospitalizations each year in infants, with more than 200 deaths attributed to RSV lower respiratory tract disease and as many 175,000 hospitalizations and 14,000 deaths in adults greater than 65 years of age each year (10, 33, 36). However, there is currently no safe and effective RSV vaccine or drug after more than 40 years of effort.

Palivizumab, targeting the RSV F protein is currently used in passive immune prophylaxis (5, 11), however passive antibody therapy for RSV is expensive, inconvenient and has limited efficacy for broader use (25). The modest prophylactic efficacy is likely linked to genetic variation in seasonal RSV strains, anti-antibody responses, and a short half-life of serum monoclonal antibodies (12, 16, 25). Thus, there is a need for development of safe and efficacious RSV vaccines. Unfortunately, natural RSV infection provides only limited protective immunity and humans may experience repeated infections and disease throughout life (2, 31). The first RSV candidate vaccine, a formalin-inactivated alum-precipitated RSV (FI-RSV) preparation, did not confer protection and was associated with a greater risk of serious disease with subsequent natural infection (6, 40). The understanding of FI-RSV enhanced diseases mechanism is still incomplete. It is thought that a deficiency in neutralizing antibodies, induction of a biased Th2-type T cell response, and poor CTL responses following vaccination contributed to the poor efficacy and enhanced pulmonary disease associated with FI-RSV vaccination (9, 26-28, 35). The experience with the FI-RSV vaccine has generated a great deal of concern about the safety of any non-live RSV vaccine and led to an extremely cautious approach to novel RSV vaccines. Increased understanding of the mechanisms of immunity and RSV disease pathogenesis are required for future RSV vaccine development, and new and effective disease intervention strategies are urgently needed for controlling RSV infection.

RSV is a single stranded negative sense RNA virus belonging to the *Pneumovirinae* subfamily in the *Pneumovirus* genus. The RSV genome contains ten genes encoding 11 viral proteins. Of the 11 viral proteins, G and F proteins are the two main surface glycoproteins which induce neutralizing antibodies (7, 34), thus they have become the focus of vaccine development. Accumulating evidence suggests that the RSV F protein is important in inducing protective immunity (15, 29), while the G protein seems to be associated with disease pathogenesis (22, 30). One of the disease mechanisms linked to the G protein induced pathologenesis is CX3C chemokine mimicry (38). RSV G protein has marked similarities and structural features to fractalkine (Fkn), the only known CX3C chemokine (38). Both G protein and Fkn exist as membrane-bound and secreted forms and both contain a CX3C chemokine motif that can bind to the fractalkine receptor, CX3CR1 (13, 21). Fractalkine functions to recruit immune cells to sites of inflammation as well as providing cell adhesion (13). In particular, Fkn mediates the recruitment and activation of CX3CR1+ leukocytes including subsets of NK cells and CD4 and CD8 T lymphocytes (19). RSV G protein has been shown to have fractalkine-like chemotactic activity *in vitro* (38). *In vivo*, acting by CX3C mimicry, RSV G protein acts as a fractalkine antagonist and modulates the immune responses by inhibiting fractalkine-mediated responses through altering the trafficking of CX3CR1+ cells and modifying the magnitude and cadence of cytokine and chemokine expression (19, 37). Infection of mice with a RSV mutant virus lacking the CX3C motif leads to a substantial increase of pulmonary NK cells and CD4 and CD8 cells compared to wild type RSV (19). This suggests that G protein CX3C-CX3CR1 interaction contributes to immune evasion and may contribute to disease pathogenesis. It is also very likely that RSV utilizes this immune modulation system to facilitate its replication and persistence.

Thus, the G protein CX3C-CX3CR1 interaction remains an important target for disease intervention strategies against RSV infection.

In the present study, we investigated a new disease intervention strategy to develop antibodies reactive to the central conserved region of CX3C motif to block its interaction with CX3CR1. We hypothesize that vaccines inducing CX3C-CX3CR1 blocking antibodies will prevent RSV G protein immune modulation and disease pathogenesis by ablating the function of CX3CR1 mimicry of RSV G protein, and lead to new strategies in vaccine development. In this study, mice were vaccinated with G protein polypeptides or peptides across different regions of the CX3C motif of RSV G protein. Our results show that antibodies specific for the central conserved region of the G protein have higher blocking activities *in vitro* and prevent body weight loss indicative of disease pathogenesis, as well as prevent lung pathology and decrease lung virus titers compared to antibodies reactive to N- and C- terminal regions of the G protein. These results suggest that a vaccine strategy to induce G protein CX3C-CX3CR1 blocking antibodies may be useful to prevent G protein immune modulation and disease pathogenesis.

Materials and Methods

Peptide and polypeptide:

G protein peptides and polypeptides spanning regions of the RSV G protein were designed for vaccination (Table 4.1 and Figure 4.2). The synthesized G peptides were dissolved in DMSO at a concentration of 2.5mg/ml. The G polypeptides were expressed and purified from *Escherichia coli*. The G gene fragments were amplified by reverse transcription-PCR, cloned into the expression vectors pQE41 or pQE42 and expressed in *Escherichia coli*. The proteins were purified by immobilized metal affinity chromatography as described previously (23).

Stably transfection of human 293 cells with CX3CR1 receptor:

Human 293 cells were transfected with the pCX3CR1 plasmid by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture protocol. Briefly, 293 cells were plated in 24-well plate at 2 x 10^5 cells/well one day before transfection to allow for 80% confluency at the time of transfection. Plasmid DNA and lipofectamine 2000 were diluted with Opti-MEM (GIBCO, Grand Island, NY) in a 96-well flat-bottom plate (Costar, Corning, NY). Diluted DNA or Lipofectamine 2000 was added into the wells and Opti-MEM was added to bring the volume up to 50μl. The diluted DNA and Lipfectamine 2000 were combined, mixed gently and incubated for 30 min at room temperature (RT) to form DNA-lipofectamin complex. Opti-MEM (300μl) was added to the complex and mixed gently. Growth medium was removed from the cells to be transfected and replaced with 200μl of growth medium without serum. The complex was then added to the cells and incubated at 37°C overnight. Growth medium (400μl) containing 2X heat-inactivated fetal bovine serum (Hyclone, Logan, Utah) was added to the cells 24h later. Cells were passaged at 1:10 dilution into selective medium DMEM (Cellgro, Herndon, VA) containing 0.5mg/ml of antibiotic (G418) (GIBCO, Grand Island, NY) out to 72 hours post-transfection. The expression of CX3CR1 was verified by flow cytometry (BD bioscience, Franklin Lakes, NJ) using FITC-conjugated anti-CX3CR1 monoclonal antibodies (MBL International, Woburn, MA).

Cell sorting:

The stably CX3CR1-transfected 293 cells (CX3CR1.293) cells were stained with FITC labeled monoclonal antibody anti-CX3CR1 (MBL International,Woburn, MA) and used for sorting high expression clones. Briefly, transfected CX3CR1.293 cells were trypsinized and resuspended in PBS (Hyclone, Logan, Utah) at 1 x 10^6 cells/ml in 15ml conical tubes BD

bioscience, Franklin Lakes, NJ). Cells were washed with washing buffer (PBS containing 0.2% BSA) at 200g for 5min and then stained with 500μl of monoclonal antibody (anti-CX3CR1-FITC 1:50 dilution) for 30min at 4°C. Cells were washed twice and resuspended in 1ml of flow buffer. Sorting was performed on a Cytomation MoFlo (Dako, Fort Collins, CO). Cells were sorted to round bottom 96-well plates (Costar, Corning, NY) filled with 200μl/well of DMEM 10% FBS culture medium with 0.5mg/ml of G418 antibiotics. Sorted cells were allowed to grow for one week to reach sufficient numbers in each well before screening for CX3CR1 expression by flow cytometry as indicated above. The highest expression clones were selected for the following fractalkine/G protein CX3C-CX3CR1 binding experiment.

RSV G protein purification:

Native G protein from RSV strain A2 was purified from RSV A2 infected Vero cells (MOI = 1) using affinity chromatography. Briefly, RSV-infected Vero E6 cells were collected at day 5 post infection and the cell pellets were resuspended in cold PBS containing 'complete' protease inhibitors (Sigma, St. Louis, MO). Triton X-100 (Sigma,St. Louis, MO) was added the cell pellet to a final concentration of 0.01%, and the cell slurry was stirred on ice for 30 minutes. Lysate was subjected to sonication at 25% power for 6 cycles of 2X 60 seconds blasts, resting for 5 minutes in between cycles, followed by centrifugation at 10,000 g for 15 min. The supernatant was then collected, filtered through a 0.2 μm filter (Whatman, Florham Park, NJ), and applied to a Hi-Trap NHS-activated column (Amersham, Piscataway, NJ) that was coupled to anti-RSV G antibody (clone 131-2G) according to the manufacturers' instructions. The column was equilibrated with 5 column volumes of PBS + 0.2% N-Octyl-β-glycoside (Thermo Scientific, Rockford, IL) using FPLC at a flow rate of 2 ml/minute. The 131-2G monoclonal antibody was used because it recognizes the G protein of RSV A2. Lysate was loaded at a flow rate of 0.5

ml/minute, the column washed with 4 column volumes of PBS + 0.2% N-Octyl-β-glycoside, and eluted with 4 column volumes of 0.1M glycine, 1% N-Octyl-β-glycoside, pH 2.2, collecting 10 X 2 ml fractions. The fractions were neutralized with 0.3 ml of 2M Tris pH 8.0, and those fractions containing the G protein (determined by UV absorption during FPLC and by Western blot analysis) were pooled and dialyzed overnight at 4°C against PBS pH 7.4. This purification yields highly purified G protein with no detectable F protein by Western blot analysis and no detergent after dialyzing.

Mice and G peptide/polypeptide vaccination:

Four-to-six-week-old specific-pathogen-free female BALB/c mice were purchased from Charles River Laboratories (Frederick, MD), housed in microisolator cages, and fed filtered water and food *ad libitum*. The studies were reviewed and approved by the university institutional review committee. All the peptides were conjugated to KLH (Pierce, Rockford, IL) for vaccination according to the manufacture protocol. KLH conjugated peptides were then used to immunize mice at the dose of 50 μg/mice together with Montanide (Seppic, Paris, France) as an adjuvant. G protein polypeptides were directly mixed with TiterMax(Norcross, Georgia) as an adjuvant and the mice were intramuscularly immunized at a dose of 50 μg/mice. Mice were boosted as indicated for primary immunization every two weeks. A modified indirect ELISA was used for the detection of antibody titers in the immune serum. The antisera from vaccinated mice were used for G protein CX3C-CX3CR1 inhibition assay.

ELISA assay:

Sera from blood of immunized mice were collected one week after the last boost. The titers of antisera were determined using a modified indirect ELISA as previously described (20). Briefly, flat-bottom ELISA plates (Costar, Corning, NY) were coated with immunizing peptides

or polypeptides overnight at 4°C. The plates were then blocked by 5% dry milk in PBS for 1 hour at 37°C .Serial of dilutions of sera in PBS were added to the wells and incubated for 1 hr at 37°C. The plates were washed three times with washing buffer (PBS containing 0.05% Tween), and incubated for 1 hr at 37°C with alkine-phosphatase conjugated goat anti-mouse IgG(H+L) (Thermo Scientific, Rockford, IL). After washing, the plates were developed with pNpp substrate (Sigma, St. Louis, MO) as described by the manufacturer.

Fractalkine-G protein CX3C-CX3CR1 binding/inhibition assay:

Antisera derived from mice vaccinated with G polypeptides and peptides were purified by immobilized protein G (Thermo Scientific, Rockford, IL) to remove endogenous CX3CL1 and other serum factors which might compete with G protein for binding to CX3CR1. Purified antisera antibodies were then pre-incubated with 500 nM of purified RSV G protein for 1 hr at 4°C. Monoclonal antibody 131-2G and protein G purified normal mouse serum were used as a positive and negative control. CX3CR1-transfected 293 cells (CX3CR1.293) were washed and plated into a round bottom 96-well plate (Costar, Corning, NY) at 2 x 10^5 cells/well. The cells were washed again and resuspended in PBS containing anti-human CD32 (Fc block) (BD bioscience, Franklin Lakes, NJ) at 1 μg/ml, and incubated at 4°C for 15 min. Cells were resuspended in the pre-mix of serum and RSV G protein + 2.5 μg/ml heparin (Neoparin Inc. Alameda, CA) to prevent non-specific binding and incubated for 45 min at 4°C. Cells were then washed in PBS containing 0.2% BSA (FACS buffer) and incubated with alexa-488 conjugated anti-G protein monoclonal antibody (130-2G) for 30 min at 4°C. The percentage of cells binding to G protein was determined by FACS analysis. The percent inhibition of G protein binding to CX3CR1 was calculated by the formula (percent specific binding to CX3CR1.293 cells in

absence of antisera antibody – percent specific binding in the presence of antisera antibody) x 100 x 100% / (percent specific binding to CX3CR1.293 cells in absence of antisera antibody).

Disease pathogenesis:

The G peptides or polypeptides used for this aspect of the study were based on their ability to induce specific antibodies in mice. G polypeptides (G1, G2, G3) and G peptides (D-1, RT32 and WT) were chosen to vaccinate mice intramuscularly for the pathogenesis study. The control groups were vaccinated with 1 x 10^6 live RSV A2 or mock Vero cell lysate intramuscularly, respectively. Mice were challenged with 1 x 10^6 live RSV A2 after the last immunization. The body weight of each mouse was measured every day after virus infection, i.e. from day 0 to day 6. Histopathological examination was performed for each group of mice after RSV infection. Lung tissues were fixed in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ), embedded in paraffin (Sigma, St. Louis, MO), sectioned, and stained with hematoxylin and eosin (Vector Laboratory, Burlingame, CA) prior to light microscopy observation. Multiple sections from each tissue block were analyzed under light microscopy.

Lung virus titers:

The RSV lung virus titers in G polypeptide or G peptide vaccinated and control mice were determined as previously described (39). Briefly, lungs were aseptically removed from mice at days 2, 4, and 6 post-RSV challenge. Individual lung samples were homogenized in 1 ml of DMEM medium by the Qiagen tissue lyser (Qiagen, Valencia, CA). The supernatants were transferred to a Cryogenic Vial (Corning, Corning, NY) and snap frozen using liquid nitrogen, and stored at –80°C until they were simultaneously assayed to minimize biological variation. For immunostaining plaque assay, ten-fold serial dilutions of the lung homogenates were added to 90% confluent Vero cell monolayers. Following adsorption for 1 hour at 37°C, cell monolayers were

overlaid with tissue culture media containing DMEM with 10% FBS and 2% carboxymethylcellulose (Sigma, St. Louis, MO), incubated at 37°C for 5 days, and the plaques enumerated by immunostaining with monoclonal antibodies against RSV F protein (clone 131-2A).

Statistics:

Student's *t* test for unpaired samples was used to compare the responses between G polypeptides/peptides immunized groups and control groups of mice. P values of <0.05 or <0.01 were considered significant.

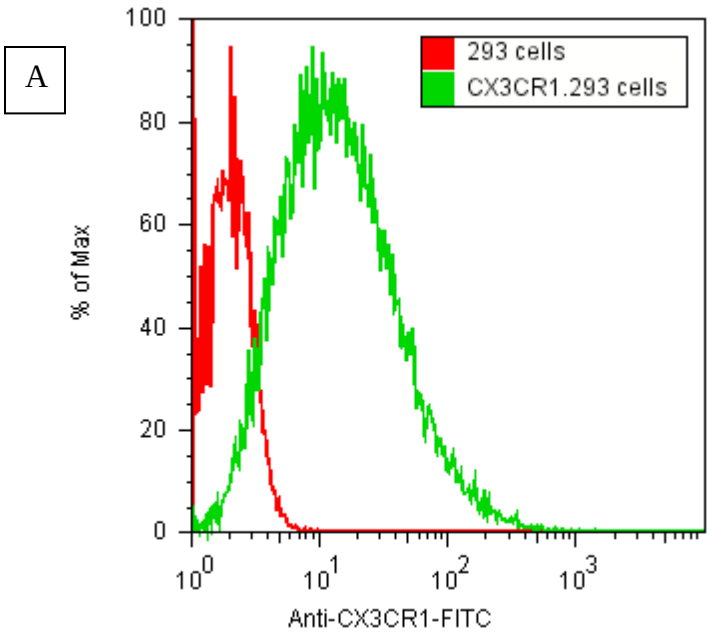
Results

G protein binds to CX3CR1 highly expressed on stably transfected human 293 cell line

CX3CR1-transfected 293 (CX3CR1.293) cells and untransfected 293 (293) cells were evaluated for G protein binding by flow cytometry. CX3CR1.293 expressing >95% CX3CR1 compared to 293 cells were used for the binding studies (Figure 4.1A). Fractalkine and RSV G protein bound to CX3CR1.293 cells in a dose-dependent manner; however, fractalkine binding had a higher apparent binding affinity compared to RSV G protein (Figure 4.1B). For example, the percent specific binding of fractalkine to CX3CR1.293 cells was similar to G protein binding but at 1000-fold lower concentrations, i.e. 100pM binds vs. 100nM, respectively. This result suggests that fractalkine has a higher affinity and/or avidity for CX3CR1 compared to G protein.

Antibody responses to G polypeptide/peptides

To determine if antibodies reactive to epitopes across the central conserved region of G protein blocked G protein interaction with CX3CR1, three G protein polypeptides and nine G peptides derived from G protein were used to vaccinate mice to generate polyclonal antibody responses. The three G protein polypeptides that were used for immunization correspond to 1)



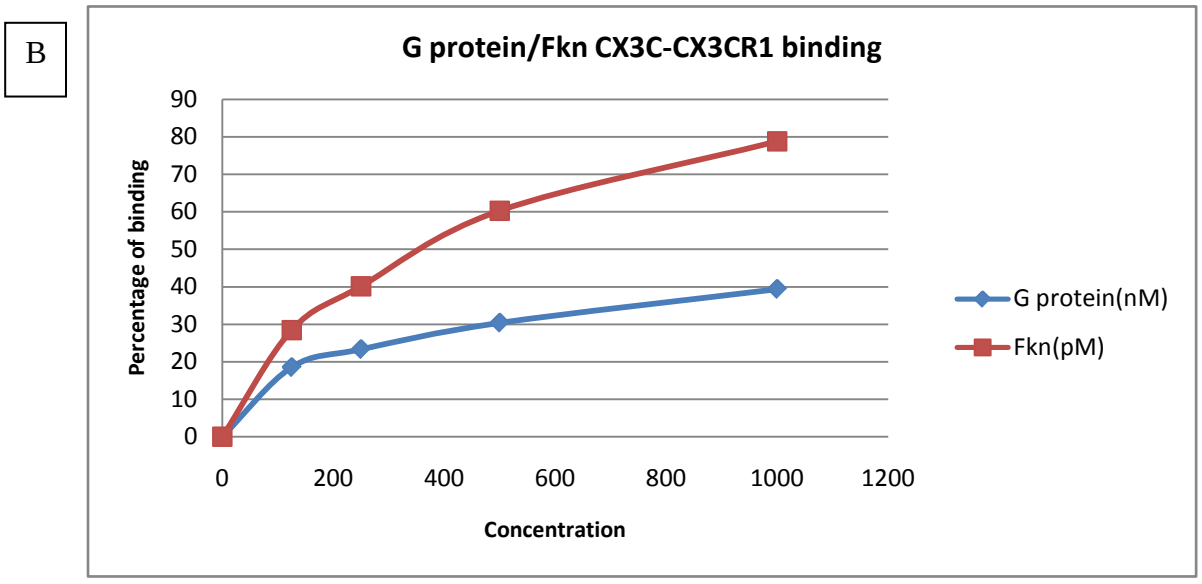


Figure 4.1. Transfection and expression of CX3CR1 receptor on human 293 cells and the binding of fractalkine and G protein to the receptor. A) A plasmid pCX3CR1 containing CX3CR1 was trasnfected into human 293 cells by lipofectamin 2000. After transfection, the cells were stained by anti-CX3CR1-FITC. Highly expression of CX3CR1 on human 293 cells after sorting was detected by flow cytometry. B) Fractalkine and RSV native G protein binds to the CX3CR1 in a dose dependent manner.

the internal variable, glycosylated region (RSV G1, aa 67-147), 2) the central conserved region (RSV G2, aa 148-198), and 3) the carboxy-terminal variable region (RSV G3, aa 199-298) of the G protein of RSV strain A2. The linear representation of the RSV G protein and localization of G polypeptides that are derived from G protein are shown in Figure 4.2. The following peptides derived from G protein were designed for vaccination (Table 4.1). 1) A wild type G protein peptide encompassing the G protein CX3C motif (AA171-201, VPCSICSNNPTCWAICKRIPNKKPGKKTTTKP; WT), 2) A 12-mer G protein peptide N-terminal to the CX3C motif (NKKPGKKTTTKP, RT32), 3) A 12-mer G protein peptide encompassing the G protein CX3C motif (TCWAICKRIPNK; GENBANK, attachment protein locus 1912305, accession number M111486, RT33) 4) A 12-mer G protein peptide C-terminal to the CX3C motif (NKKPGKKTTTKP, RT34) , 5) A 12-mer G protein peptide variant of RT33 containing a ALA substitution for ILE in the CX3C motif (TCAAACKRIPNK, RT+ala), 6) A 12-mer G protein peptide with a deletion of ILE in the CX3C motif (TCWACKRIPNKK, D-1), 7) A 12-mer G protein peptide with an ALA addition in the CX3C motif (TCWAIACKRIPN, D+1) , 8) a heterologous I-Ed-restricted 12-mer peptide from the L protein (amino acids 393-405) of RSV, and 9) a G peptide that has previously been shown to induce a protective immune response (amino acids 174-187) with the substitution Cys186→Ser (32). Mice were vaccinated with 50 μg of polypeptide or peptide antigens and boosted every two weeks; however for G polypeptides, mice were boosted once and for G peptides twice. Seven days after the last immunization, all three G polypeptides used for vaccination generated substantial antibody responses in which G2 polypeptide produced highest antibody response (Figure 4.3A). However, the peptides gave differential antibody responses, and within the nine peptides examined, three

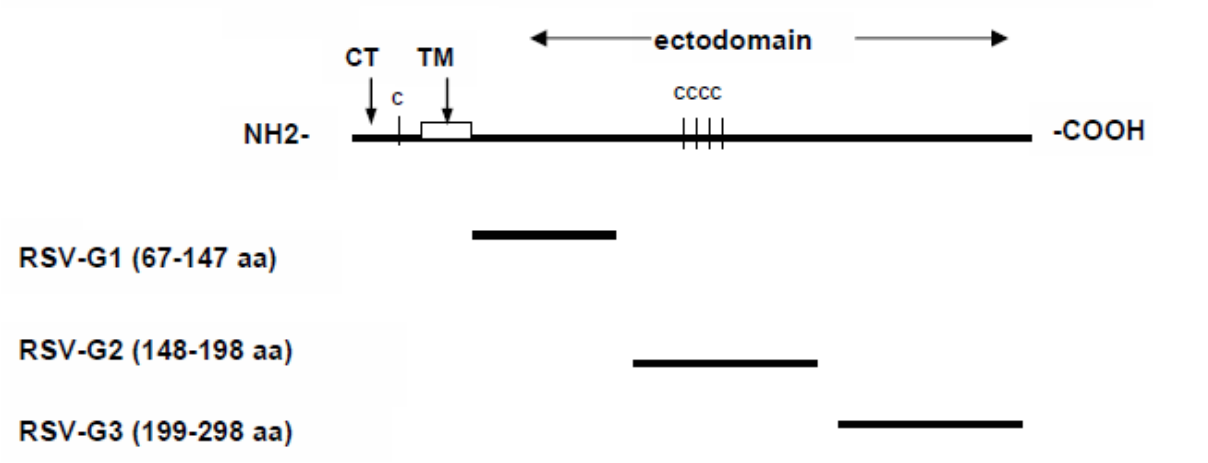


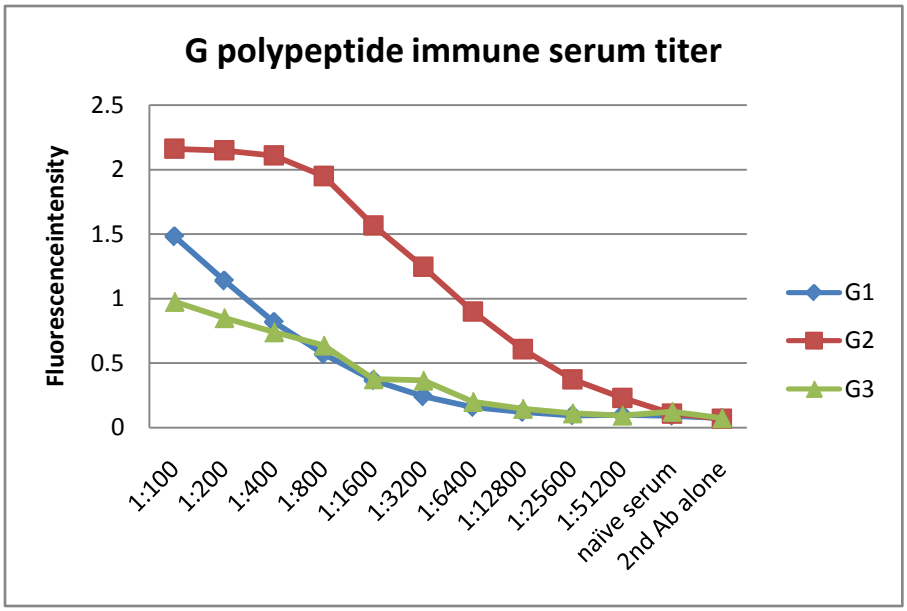
Figure 4.2. Linear representation of the RSV G protein and G fragments. The cysteines found in the G protein are indicated as C, while the transmembrane and cytoplasmic domains are indicated by TM and CT, respectively.

Table 4.1. The G peptides designed to immunize mice for antisera

Peptide	**Partial G glycoprotein AA sequence**
	171 182 186 201
WT	VPCSICSNNPTCWAICKRIPNKKPGKKTTTKP
RT32	VPCSICSNNPTC
RT33	TCWAICKRIPNK
RT34	NKKPGKKTTTKP
RT+ala	TCAAACKRIPNK
D-1	TCWA CKRIPNKK
D+1	TCWAIACKRIPNK
L393	INGKWIILLSKF
G174-187	SICSNNPTCWAICK

The location of the CX3C motif(CWAIC) in the G glycoprotein is compared to G glycoprotein peptides outside the CX3C motif(RT32 and RT34), containing the CX3C motif (RT33) or to peptides with a single AA deletion (D-1) or insertion (D+1)

A



B

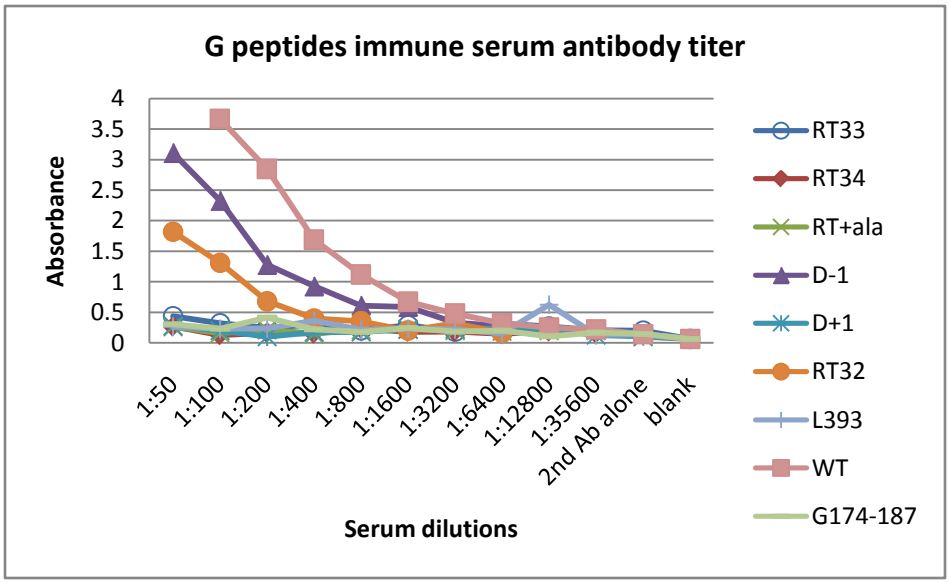


Figure 4.3. The titer of immune sera from mice immunized by G polypeptides and peptides. A) Mice were immunized with three G polypeptides(G1, G2 and G3) mixed with TiterMax at 50ug antigen per mice. Two weeks later, the mice were boosted by the same antigens. Blood were collected seven days after the last immunization and antibody titers in the sera were measured by ELISA assay. B) Mice were immunized with nine G peptides(indicated in Table1) mixed with Montanide at 50 μg antigen per mice. At two weeks interval, the mice were boosted twice by the same antigens. Blood were collected seven days after the last immunization and antibody titers in the sera were measured by ELISA assay.

peptides (WT, RT32 and D-1) produced substantial antibody responses (Figure 4.3B). It is likely that these three peptides contain immunodominant conserved linear epitopes.

Antisera inhibition of G protein binding to CX3CR1

Antisera derived from three polypeptides (G1, G2 and G3) and three peptides (WT, RT32 and D-1) that reacted well to G protein (Figure 4.3) were used to determine their ability to block G protein binding to CX3CR1 (Figure 4.4). The results indicated that antibodies specific for N- and C- terminal regions across the central conserved region of the G protein have different blocking activities; however, G2 specific antibodies have the highest inhibition (Figure 4.4). As expected, antibodies reactive to the WT peptide had higher inhibition compared to the RT32 and D-1. These results demonstrated that CX3C motif in the G protein is important for the CX3CR1 receptor binding, and that antibodies recognizing epitopes N- or C-terminal proximal to the CX3C motif have a reduced ability to block G protein binding to CX3CR1 receptor.

G polypeptide/peptide vaccination reduce disease pathogenesis in mice

Mice were vaccinated intramuscularly with G protein polypeptides (G1, G2 and G3) and peptides (D-1, RT32 and WT) to determine the association between antibodies that block G binding to CX3CR1 and inhibit or reduce RSV disease severity following RSV challenge (Figure 4.3). The live RSV vaccination and no vaccination were added as controls. The antibody titers in the vaccinated mice were tested against the immunizing antigens or RSV. After RSV challenge of vaccinated mice, weight loss at day from day 0 to day 6 post-infection was determined as one parameter of disease pathogenesis. In addition, the lungs from the vaccinated mice were examined for histopathology by H&E staining. The results showed that mice vaccinated with G protein polypeptides or peptides that induced G protein CX3C-CX3CR1 blocking antibodies displayed less body weight loss compared to control vaccinated mice or unvaccinated mice

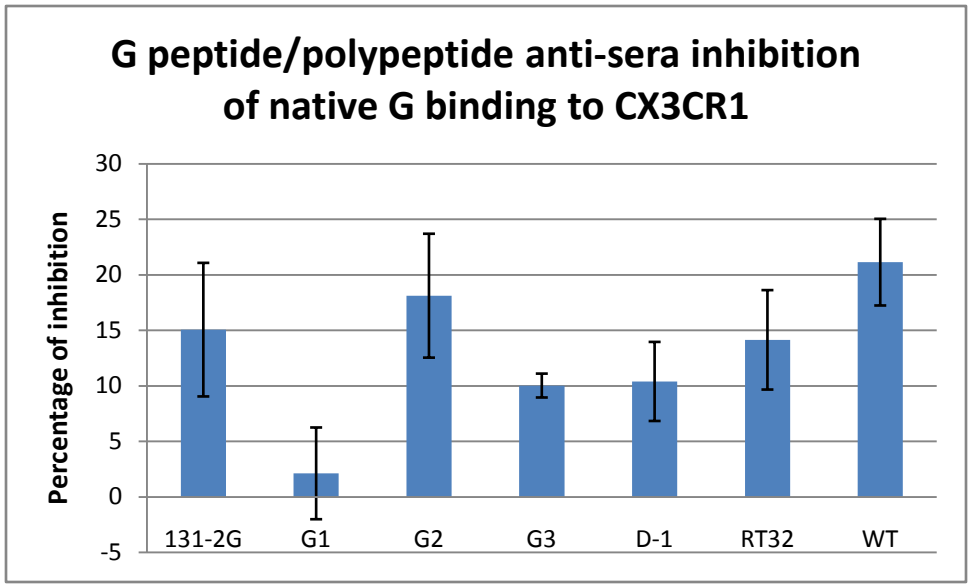


Figure 4.4. The purified antisera antibody derived from mice vaccinated with G protein polypeptides and peptides prevent native RSV G protein binding to CX3CR1 to different levels. The native G protein was first pre-incubated with antisera and 2.5 μg/ml of heparin for 45min at 4°C. CX3CR1.293 cells were blocked with Fc-block and incubated with G protein-antisera pre-mix for 45min at 4°C. Then the cells were stained with anti-G mAb(130-2G) conjugated with Alexa488 for the detection of binding of G protein to CX3CR1 by flow cytomety.

(Figure 4.5). Within the groups of vaccinated mice, the G2 polypeptide and WT peptide vaccinated mice showed the least body weight loss compared to any of the other vaccination groups (Figure 4.5). Importantly these results are consistent with these vaccinated mice also generating the highest G protein CX3C-CX3CR1 blocking antibodies (Figure 4.4). The levels of peribronchiolar, perivascular, and interstitial lymphocytic infiltrates is typical in RSV-mediated pathogenesis (14). No lung histopathology was evident at day 0 prior to RSV infection for any vaccination group, which indicated that the vaccination itself did not cause obvious lung pathogenesis (Figure 4.6 a, d, g, j, m, p, s and v,). Substantially reduced lung pathology was observed in both G2 polypeptides and WT peptide vaccination group at day 4 and day 6 post infections compared to any of the other vaccination groups (Figure 4.6 g-i and s-u). These results indicate that G protein CX3C-CX3CR1 interaction is an important mechanism contributing to RSV-mediated disease pathogenesis. These results suggest that RSV vaccines that induce CX3C-CX3CR1 blocking antibodies may reduce the pathology of diseases in mice.

G polypeptides/peptide vaccination reduces lung virus replication.

Lung virus titers were determined at days 2, 4 and 6 post-RSV challenge of G peptide or polypeptide vaccinated mice (Figure 4.7). Peak lung virus titers were detected at day 4 post infection in all vaccinated groups, and surprisingly, mice vaccinated with live RSV showed the highest virus titer at day 4 post-infection compared to other peptide or polypeptide vaccinated groups (Figure 4.7). The G2 polypeptide and WT peptide vaccinated mice had lowest virus titers of all the vaccinated mice at every time point post-infection, and significantly ($P<0.05$) lower virus titers compare to any of the vaccinated mice at day 4 post-infection. These results show that vaccines that induce CX3C-CX3CR1 blocking antibodies can reduce virus replication in mice. It is possible that the higher lung virus titers in the live RSV vaccinated mice may reflect immune dysregulation potentially through CX3C chemokine mimicry.

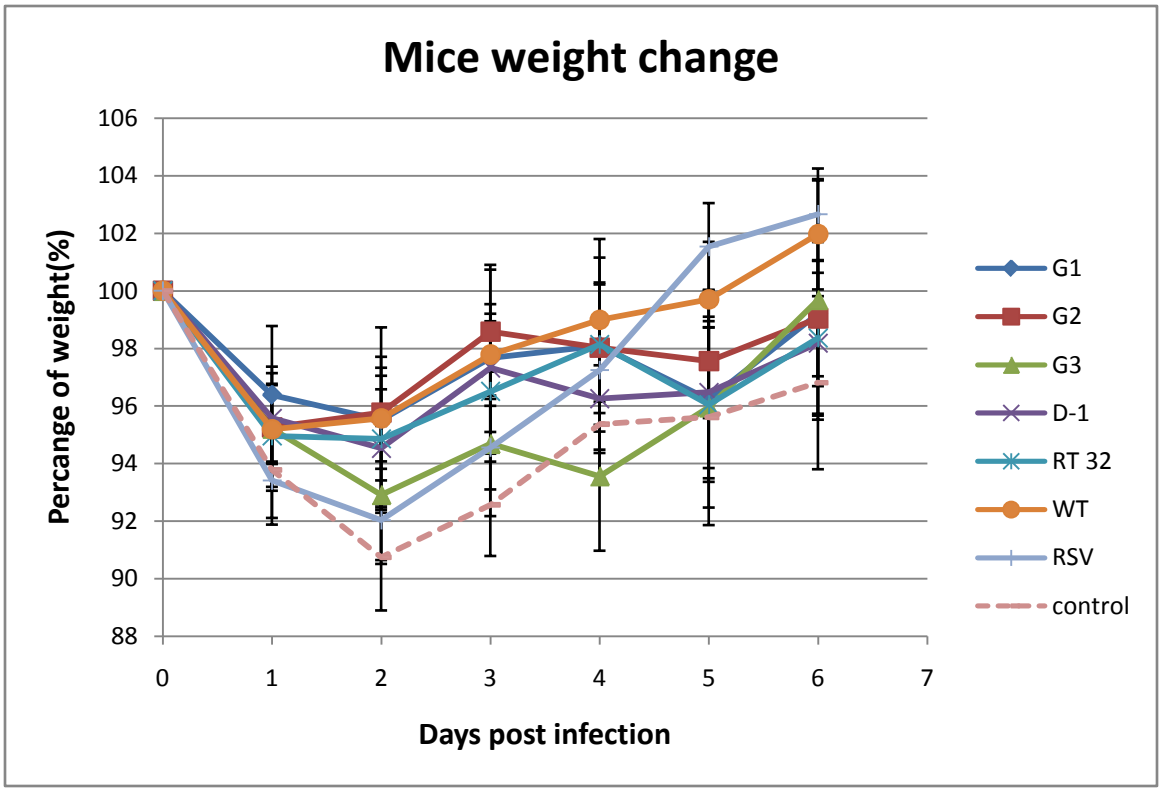


Figure 4.5. The weight change of mice vaccinated with G protein polypeptide, peptides, RSV and non-vaccinated control. Mice were vaccinated with G protein polypeptides (G1, G2 and G3) or KLH conjugated peptides (D-1, RT32 and WT) and boosted every two weeks. For polypeptides, mice were boosted once and for peptides, twice. Then mice were infected with 1 x 10^6 PFU/mice of RSV A2.

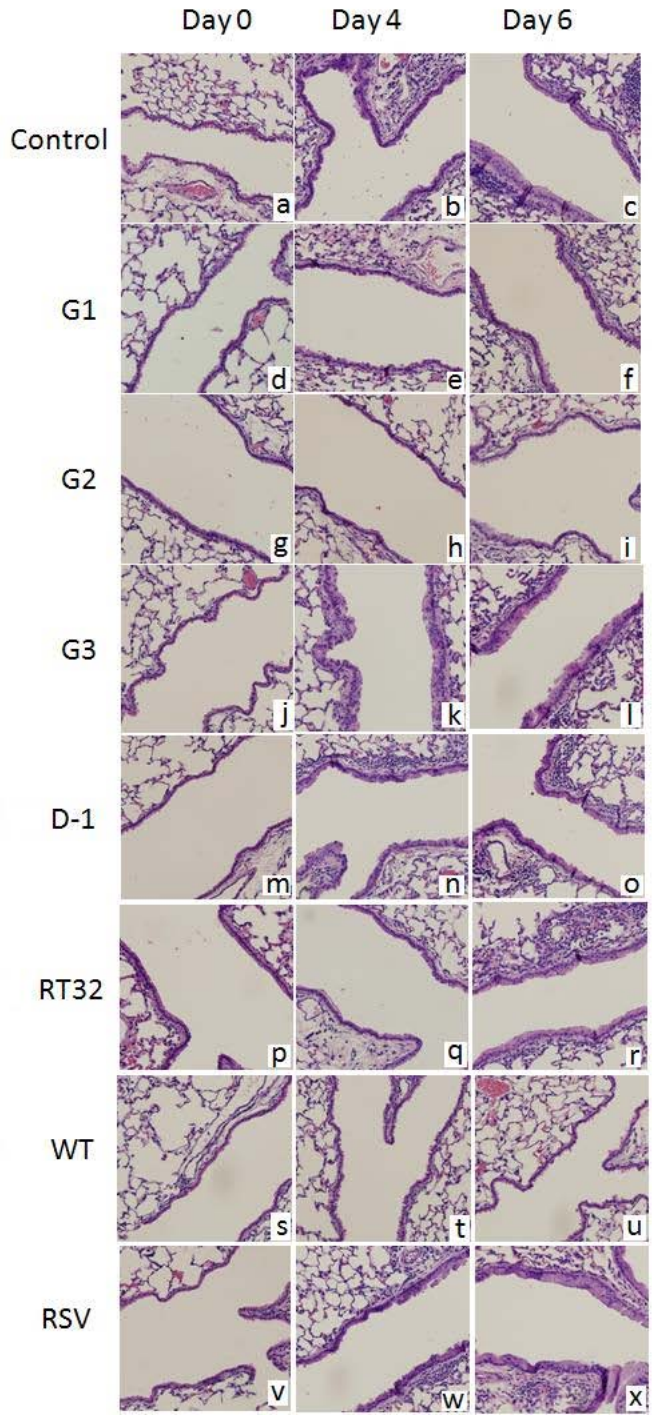


Figure 4.6. The lung histopathology of mice vaccinated with G protein polypeptide, peptides, live RSV and non-vaccinated control. Mice were vaccinated with G protein polypeptides(G1,G2 and G3) or KLH conjugated peptides(D-1, RT32 and WT) and live RSV or no vaccination control. Mice were boosted every two weeks. For polypeptides, mice were boosted once and for peptides, twice. Then mice were infected with 1 x 10^6 PFU/mice of RSV A2 seven days after the last vaccination. Lung histopathology was examined by H&E staining at day 0, 4 and 6 post infections.

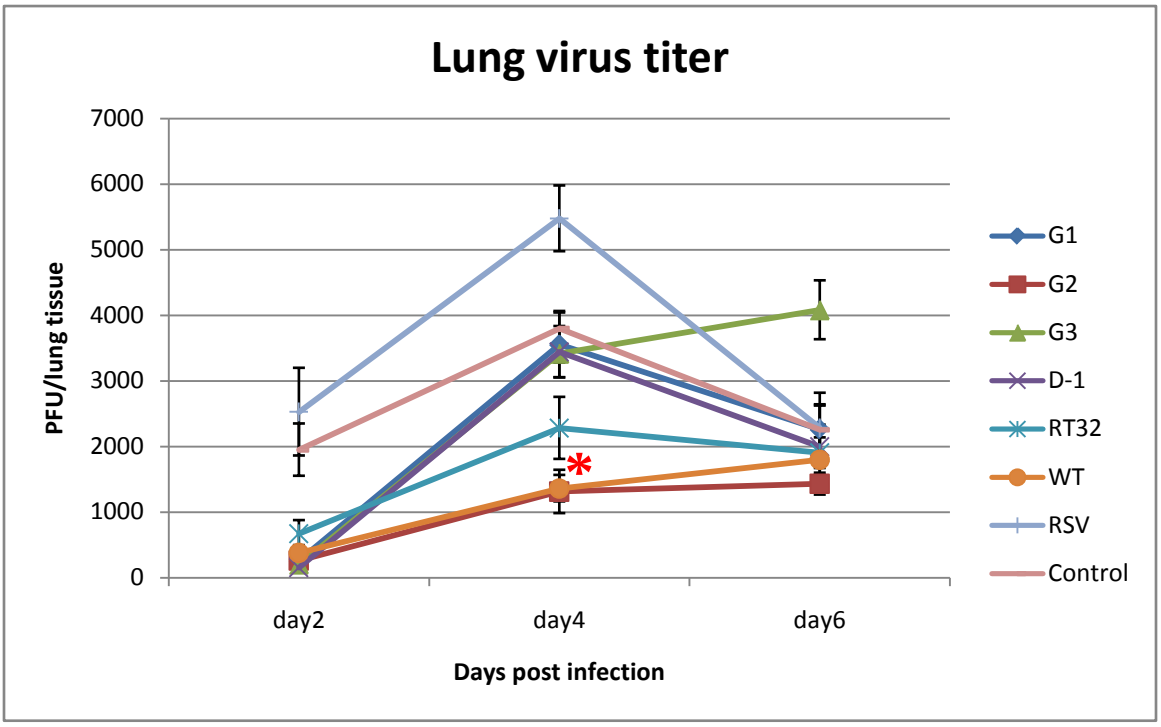


Figure 4.7. Lung virus titers in mice vaccinated with G protein polypeptide, peptides, live RSV and non-vaccinated control. Mice were vaccinated with G protein polypeptides (G1, G2 and G3) or KLH conjugated peptides (D-1, RT32 and WT) and live RSV or no vaccination control. Mice were boosted every two weeks. For polypeptides, mice were boosted once and for peptides, twice. Then mice were infected with 1 x 10^6 PFU/mice of RSV A2 7 days after last vaccination. Lung virus titers were detected by immunostaining plaque assay at day 2, 4 and 6 post infections.

Discussion

The RSV G and F proteins are the two major viral surface proteins responsible for inducing the majority of the immune response to RSV (1, 29). Notably, the F protein can induce neutralizing antibodies and a level of protective immunity; however, the G protein induces limited or no neutralizing antibodies and appears to have an important role in modulating immunity and is linked with RSV disease pathogenesis. The RSV G protein contains a CX3C chemokine motif in the central conserved region of the protein that mimics the activities of fractalkine, the only CX3C chemokine, by binding to the CX3C receptor and antagonizing the activities of fractalkine (38). Besides modulating CX3C chemokine activity, several studies have shown that the G protein also contributes to immune evasion by modifying Th1 and Th2-type cytokine and other chemokine responses, particularly the MIP family of chemokines (3, 19, 38).

In this study, we evaluated RSV G protein binding to CX3CR1 expressed on human 293 cells lines, and antibody blocking of G protein-CX3CR1 interaction by vaccination studies with G protein peptides or polypeptides. The studies demonstrated that antibodies directed to or immediately proximal to the CX3C site in the G protein can block binding to CX3CR1, reduce lung virus titers, and reduce disease pathogenesis. The results showed that CX3C motif in the G protein is important for the CX3CR1 receptor binding and antibodies against CX3C motif can inhibit G protein binding to CX3CR1 receptor. The central conserved G protein polypeptide (G2) and peptide (WT) were able to induce potent and specific antibodies having higher G protein blocking activities compared to antibodies induced by N-terminal (G1) or C-terminal (G3) polypeptides. Interestingly, the study showed that a single amino acid deletion from CX3C motif in a peptide (D-1) dramatically reduces the ability antibodies generated to this peptide to block G protein binding to CX3CR1, thus indicating a critical amino acid in the antibody epitope.

Examining some parameters of disease pathogenesis in mice vaccinated with G polypeptide or peptide vaccines revealed that disease protection correlated with antibodies that blocked G protein-CX3CR1 binding as determined *in vitro* . In these studies, the best protection from weight loss or lung histopathology was related to vaccination with the G2 polypeptide or WT peptides that were derived from the CX3C conserved region of RSV G protein. Interestingly, mice vaccinated with live RSV and subsequently challenged with live RSV had the highest levels of enhanced lung disease pathology compared to any of the other vaccination groups or to the no vaccination control. This indicates that antibodies generated to the initial virus vaccination were generally ineffective from protecting from challenge with the same strain of virus (a feature commonly observed in humans) suggesting immune modification by the G protein and likely G protein CX3C-CX3CR1 interaction may contribute to the disease pathogenesis observed. These results indicate that RSV G protein may play a role in disease pathogenesis and CX3C chemokine mimicry may contribute the enhanced disease pathogenesis as mice vaccinated with peptides or polypeptides that induce CX3C-CX3CR1 blocking antibodies exhibit reduced pathology of diseases.

Of note, the results also showed that vaccines that induce CX3C-CX3CR1 blocking antibodies induce antibodies that effectively reduce lung virus replication. Studies examining sera from all vaccinated groups indicated that antibodies with virus neutralization were only generated from mice vaccinated with peptides or polypeptides encompassing the G protein CX3C site. This is consistent with studies in the literature indicating that very few G protein-specific monoclonal antibodies efficiently neutralize RSV, and that G protein-specific antibody neutralization requires multiple antibodies (24). It is likely that the higher lung virus titers in the live RSV vaccinated mice reflect immune dysregulation. Beyond the ability of antibodies

directed to the G protein CX3C site to neutralize RSV, it is also very likely that blocking G protein CX3C-CX3CR1 interaction may facilitate infiltration of CX3CR1+ NK cells, CD4 and CD8 T cells into the lungs leading to faster virus clearance.

The outcome from the studies have suggested that modifying the G protein CX3C motif or inducing antibodies that block G protein CX3C-CX3CR1 interaction should enhance vaccine safety and efficacy and offer a promising new strategy for RSV vaccine development.

Acknowledgements

We thank Yi Kuang for her assistance in H&E staining of mice lung samples, Jamie Barber for his discussions on flow cytometry and support from NIH grant 5R01AI069275-03.

References

1. **Bembridge, G. P., N. Rodriguez, R. Garcia-Beato, C. Nicolson, J. A. Melero, and G. Taylor.** 2000. Respiratory syncytial virus infection of gene gun vaccinated mice induces Th2-driven pulmonary eosinophilia even in the absence of sensitisation to the fusion (F) or attachment (G) protein. Vaccine **19:**1038-46.

2. **Black, C. P.** 2003. Systematic review of the biology and medical management of respiratory syncytial virus infection. Respir Care **48:**209-31; discussion 231-3.

3. **Cepika, A. M., A. Gagro, A. Bace, D. Tjesic-Drinkovic, J. Kelecic, T. Baricic-Voskresensky, M. Matic, V. Drazenovic, I. Marinic, G. Mlinaric-Galinovic, Z. Vrtar, and S. Rabatic.** 2008. Expression of chemokine receptor CX3CR1 in infants with respiratory syncytial virus bronchiolitis. Pediatr Allergy Immunol **19:**148-56.

4. **Chanock, R. M., R. H. Parrott, M. Connors, P. L. Collins, and B. R. Murphy.** 1992. Serious respiratory tract disease caused by respiratory syncytial virus: prospects for improved therapy and effective immunization. Pediatrics **90:**137-43.

5. **Chavez-Bueno, S., A. Mejias, R. A. Merryman, N. Ahmad, H. S. Jafri, and O. Ramilo.** 2007. Intravenous palivizumab and ribavirin combination for respiratory syncytial virus disease in high-risk pediatric patients. Pediatr Infect Dis J **26:**1089-93.

6. **Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette.** 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am J Epidemiol **89:**449-63.

7. **Connors, M., P. L. Collins, C. Y. Firestone, and B. R. Murphy.** 1991. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J Virol **65:**1634-7.

8. **Crowe, J. E., Jr., and J. V. Williams.** 2003. Immunology of viral respiratory tract infection in infancy. Paediatr Respir Rev **4:**112-9.

9. **De Swart, R. L., T. Kuiken, H. H. Timmerman, G. van Amerongen, B. G. Van Den Hoogen, H. W. Vos, H. J. Neijens, A. C. Andeweg, and A. D. Osterhaus.** 2002. Immunization of macaques with formalin-inactivated respiratory syncytial virus (RSV) induces interleukin-13-associated hypersensitivity to subsequent RSV infection. J Virol **76:**11561-9.

10. **Falsey, A. R., and E. E. Walsh.** 1998. Relationship of serum antibody to risk of respiratory syncytial virus infection in elderly adults. J Infect Dis **177:**463-6.

11. **Feltes, T. F., and H. M. Sondheimer.** 2007. Palivizumab and the prevention of respiratory syncytial virus illness in pediatric patients with congenital heart disease. Expert Opin Biol Ther **7:**1471-80.

12. **Fenton, C., L. J. Scott, and G. L. Plosker.** 2004. Palivizumab: a review of its use as prophylaxis for serious respiratory syncytial virus infection. Paediatr Drugs **6:**177-97.

13. **Fong, A. M., L. A. Robinson, D. A. Steeber, T. F. Tedder, O. Yoshie, T. Imai, and D. D. Patel.** 1998. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. J Exp Med **188:**1413-9.

14. **Graham, B. S., M. D. Perkins, P. F. Wright, and D. T. Karzon.** 1988. Primary respiratory syncytial virus infection in mice. J Med Virol **26:**153-62.

15. **Groothuis, J. R., S. J. King, D. A. Hogerman, P. R. Paradiso, and E. A. Simoes.** 1998. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PFP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J Infect Dis **177:**467-9.

16. **Groothuis, J. R., and H. Nishida.** 2002. Prevention of respiratory syncytial virus infections in high-risk infants by monoclonal antibody (palivizumab). Pediatr Int **44:**235-41.

17. **Hacking, D., and J. Hull.** 2002. Respiratory syncytial virus--viral biology and the host response. J Infect **45:**18-24.

18. **Hall, C. B.** 1999. Respiratory syncytial virus: A continuing culprit and conundrum. J Pediatr **135:**2-7.

19. **Harcourt, J., R. Alvarez, L. P. Jones, C. Henderson, L. J. Anderson, and R. A. Tripp.** 2006. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. J Immunol **176:**1600-8.

20. **Harcourt, J. L., R. A. Karron, and R. A. Tripp.** 2004. Anti-G protein antibody responses to respiratory syncytial virus infection or vaccination are associated with inhibition of G protein CX3C-CX3CR1 binding and leukocyte chemotaxis. J Infect Dis **190:**1936-40.

21. **Hendricks, D. A., K. Baradaran, K. McIntosh, and J. L. Patterson.** 1987. Appearance of a soluble form of the G protein of respiratory syncytial virus in fluids of infected cells. J Gen Virol **68 (Pt 6):**1705-14.

22. **Johnson, T. R., J. E. Johnson, S. R. Roberts, G. W. Wertz, R. A. Parker, and B. S. Graham.** 1998. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. J Virol **72:**2871-80.

23. **Jones, L. P., H. Q. Zheng, R. A. Karron, T. C. Peret, C. Tsou, and L. J. Anderson.** 2002. Multiplex assay for detection of strain-specific antibodies against the two variable regions of the G protein of respiratory syncytial virus. Clin Diagn Lab Immunol **9:**633-8.

24. **Melero, J. A., B. Garcia-Barreno, I. Martinez, C. R. Pringle, and P. A. Cane.** 1997. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. J Gen Virol **78 (Pt 10):**2411-8.

25. **Moler, F. W., R. W. Brown, R. G. Faix, and J. R. Gilsdorf.** 1999. Comments on palivizumab (Synagis). Pediatrics **103:**495-7.

26. **Murphy, B. R., G. A. Prince, P. L. Collins, K. Van Wyke Coelingh, R. A. Olmsted, M. K. Spriggs, R. H. Parrott, H. W. Kim, C. D. Brandt, and R. M. Chanock.** 1988. Current approaches to the development of vaccines effective against parainfluenza and respiratory syncytial viruses. Virus Res **11:**1-15.

27. **Murphy, B. R., G. A. Prince, E. E. Walsh, H. W. Kim, R. H. Parrott, V. G. Hemming, W. J. Rodriguez, and R. M. Chanock.** 1986. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J Clin Microbiol **24:**197-202.

28. **Nicholas, J. A., K. L. Rubino, M. E. Levely, E. G. Adams, and P. L. Collins.** 1990. Cytolytic T-lymphocyte responses to respiratory syncytial virus: effector cell phenotype and target proteins. J Virol **64:**4232-41.

29. **Olmsted, R. A., N. Elango, G. A. Prince, B. R. Murphy, P. R. Johnson, B. Moss, R. M. Chanock, and P. L. Collins.** 1986. Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc Natl Acad Sci U S A **83:**7462-6.

30. **Openshaw, P. J.** 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am J Respir Crit Care Med **152:**S59-62.

31. **Power, U. F.** 2008. Respiratory syncytial virus (RSV) vaccines--two steps back for one leap forward. J Clin Virol **41:**38-44.

32. **Simard, C., F. Nadon, C. Seguin, N. N. Thien, H. Binz, J. Basso, J. F. Laliberte, and M. Trudel.** 1997. Subgroup specific protection of mice from respiratory syncytial virus infection with peptides encompassing the amino acid region 174-187 from the G glycoprotein: the role of cysteinyl residues in protection. Vaccine **15:**423-32.

33. **Simoes, E. A.** 2003. Environmental and demographic risk factors for respiratory syncytial virus lower respiratory tract disease. J Pediatr **143:**S118-26.

34. **Stott, E. J., G. Taylor, L. A. Ball, K. Anderson, K. K. Young, A. M. King, and G. W. Wertz.** 1987. Immune and histopathological responses in animals vaccinated with recombinant vaccinia viruses that express individual genes of human respiratory syncytial virus. J Virol **61:**3855-61.

35. **Tang, Y. W., and B. S. Graham.** 1994. Anti-IL-4 treatment at immunization modulates cytokine expression, reduces illness, and increases cytotoxic T lymphocyte activity in mice challenged with respiratory syncytial virus. J Clin Invest **94:**1953-8.

36. **Thompson, W. W., D. K. Shay, E. Weintraub, L. Brammer, N. Cox, L. J. Anderson, and K. Fukuda.** 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA **289:**179-86.

37. **Tripp, R. A., A. Dakhama, L. P. Jones, A. Barskey, E. W. Gelfand, and L. J. Anderson.** 2003. The G glycoprotein of respiratory syncytial virus depresses respiratory rates through the CX3C motif and substance P. J Virol **77:**6580-4.

38. **Tripp, R. A., L. P. Jones, L. M. Haynes, H. Zheng, P. M. Murphy, and L. J. Anderson.** 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol **2:**732-8.

39. **Tripp, R. A., D. Moore, L. Jones, W. Sullender, J. Winter, and L. J. Anderson.** 1999. Respiratory syncytial virus G and/or SH protein alters Th1 cytokines, natural killer cells, and neutrophils responding to pulmonary infection in BALB/c mice. J Virol **73:**7099-107.

40. **Weibel, R. E., J. Stokes, Jr., M. B. Leagus, C. C. Mascoli, and M. R. Hilleman.** 1966. Respiratory virus vaccines. V. Field evaluation for efficacy of heptavalent vaccine. Am Rev Respir Dis **94:**362-79.

CHAPTER 5

CONCLUSION

Despite the importance of respiratory syncytial virus (RSV) as a leading agent of severe respiratory diseases in infants, young children, and the elderly there is still no safe and effective vaccine and treatments are limited. To address these issues two studies were investigated: 1) a RNAi therapeutic drug approach to reduce RSV disease pathogenesis while allowing for effective immunization, and 2) evaluating RSV G protein subunit vaccines composed of G protein peptides or polypeptides to induce antibodies that block G protein CX3C-CX3CR1 interaction.

The first study centered on the development of siRNA therapeutics investigated how RNA interference can facilitate vaccine development. Specifically, we examined the effect of prophylactic siRNA treatment targeting the RSV P gene on the primary and memory immune response to RSV infection or challenge. In this study, we used small inhibitory RNA (siRNA) targeting a conserved viral gene to inhibit RSV replication in host. The purpose, however, was not to completely halt the replication of virus, but to reduce pathogenesis and expose the immune system to sufficient virus so as to vaccinate against future challenges. There are several important findings related to this study. First, we have shown that virus load is relevant to the outcome of the memory response. Our results indicated that siRNA prophylaxis without reducing the initial virus load induces better memory responses than the treatment of initial low virus load. Second, we have shown that reduced virus load in lung is associated with reduced inflammatory response, which tested our hypothesis that the virus load contributes to disease severity in part

due to the host immune response. However, pathogenesis does not appear to be directly linked to the RSV-specific $CD8^+$ T cell response because WT siRNA treated mice have higher numbers of RSV M2-specific $CD8^+$ T cells and have low levels of pulmonary pathogenesis compared to MM siRNA or PBS treated mice. On the other hand, RSV M2-specific $CD8^+$ T cells have been shown to regulate and reduce Th2-mediated pathology in an IFNγ-independent manner, which is consistent with limited level of lung histopathology, observed in WT siRNA treated mice compared to MM siRNA or PBS treated mice. Third, our study has revealed the possibility that reduced virus load related to WT siRNA treatment limits the threshold required for some RSV genes to modify aspects of RSV immunity leading to a more appropriate anti-viral response. In summary, our results in this study showed that siRNA can act as effective anti-viral drug to reduce the viral load and disease pathogenesis while allowing for the induction of a potent memory response to challenge. The findings from these studies provide a foundation for new RSV disease intervention studies that employ RNAi technologies, and may offer a new direction in treatments for RSV

The second area of study was centered on the development of therapeutic antibodies against RSV infection. RSV can envade immunity via various modulatory mechanisms; however RSV G protein CX3C chemokine mimicry has been shown to be very important in facilitating RSV infection and replication. Thus, the studies determined the regions in the G protein that induce G protein CX3C-CX3CR1 blocking antibodies to help identify important vaccine components and therapeutic antibody approaches. All three G polypeptides used for vaccination produced good antibody responses in which G2 polypeptide produced highest antibody response. The peptides we designed to immunize the mice have different humoral immunogenicity. Within the nine peptides, three peptides (WT, RT32 and D-1) produce good antibody responses after

two boosts. The study showed that antibodies specific for N- and C- terminal regions and across the central conserved region of the G protein have different CX3C-CX3CR1 blocking activities. G2 specific antibodies directed against the central conserved region had the highest inhibition of G protein binding to CX3CR1, i.e. ~ 30%. Antibodies induced by WT peptide vaccination had higher inhibition compared to the RT32 and D-1 peptide vaccinated mice. The one amino acid deletion clearly reduced the blocking activity. These results demonstrate that the CX3C motif in the G protein is important for the CX3CR1 receptor binding. Consistent with the *in vitro* experiments, the *in vivo* experiments in mice showed that vaccination with G polypeptide or peptide vaccines that induce antibodies with CX3C-CX3CR1 blocking activities have reduced lung disease pathogenesis compared to the control or no vaccination and to live RSV vaccination. Correspondingly, G polypeptide or peptide vaccines that induce antibodies with CX3C-CX3CR1 blocking activities reduce lung disease pathogenesis and these vaccination groups also have the lowest lung virus titers among all vaccination groups. Together, these data results show that protective anti-G protein antibodies recognizing the central conserved cysteine-rich region of the G protein prevent RSV disease pathogenesis, which is linked to antibody-mediated inhibition of G protein CX3C interaction with CX3CR1 and immune modulation.

In summary, these studies have defined new strategies for safe and effective RSV vaccines, and aided our understanding of some of the mechanisms contributing to RSV disease in humans.