RESPIRATORY SYNCYTIAL VIRUS DISEASE INTERVENTION:

DEVELOPMENT OF RSV G POLYPEPTIDE-BASED SUBUNIT VACCINES

by

YOUNGJOO CHOI

(Under the Direction of RALPH TRIPP)

ABSTRACT

Respiratory syncytial virus (RSV) is a single-stranded, negative sense RNA virus in the Paramyxovirus family that can cause serious lower respiratory tract disease in infants, young children, and the elderly. Currently no safe and effective RSV vaccine exists due to the difficulties in balancing vaccine efficacy and safety. Previous studies have shown that RSV can modify the host immune response via RSV G protein CX3C chemokine mimicry, adversely affecting pulmonary leukocyte chemotaxis, $CX3CR1^{+}$ RSV-specific T cell responses, and cytokine and chemokine expression. Accordingly, we investigated whether vaccination of mice with RSV G protein-derived polypeptides can induce neutralizing and/or blocking antibodies capable of inhibiting G protein-CX3CR1 interaction for both A and B strains of RSV. Our results examining G polypeptide and G nanoparticle vaccination of mice show that these RSV G polypeptide subunit vaccines generate heterosubtypic neutralizing antibodies that block the

immune modulatory activities of RSV A2 and B1 G protein and G protein interaction with CX3CR1.

INDEX WORDS: RSV, Vaccine, G protein, G glycoprotein, CX3CR1, Nanoparticle, Microparticle, Subunit, Paramyxovirus, Virus

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YOUNGJOO CHOI

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YOUNGJOO CHOI

Major Professor:	Ralph A. Tripp
Committee:	Stephen M. Tompkins
	Biao He
	Donald A. Harn
	Wendy Watford.

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I would like to dedicate this dissertation to my mother, who, for sixteen years, has endured the absence of her son from home.

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CHAPTER 1

INTRODUCTION

Respiratory syncytial virus (RSV) is a common cause of serious lower respiratory tract disease in infants, young children, and the elderly worldwide (2-4, 8). RSV is classified into two major groups: strains A and B, which primarily differ in the amino acid composition of their attachment glycoprotein (G protein). Natural infection with RSV provides limited long-term protection from reinfection and disease, as evidenced by the recurrence of even severe RSV infections throughout life (7, 13). Despite numerous efforts to develop RSV vaccines, none have been shown to be safe and effective. The challenges that impede the development of safe and effective RSV vaccines have been associated with the virus's ability to modify various aspects of the host immune response. An important viral mechanism shown to be involved in the modification of host immunity is CX3C chemokine mimicry mediated by the RSV G protein (26). The central conserved region of the G protein in both RSV A and B strains consists of a CX3C chemokine motif that has been shown to mimic the activities of fractalkine (CX3CL), the only known member of the CX3C chemokine family (26). Fractalkine, as well as the RSV G protein, exists as both membrane-anchored and secreted forms (16-18, 23). While membrane-anchored fractalkine serves as an adhesion molecule for the leukocytes on the activated endothelial cells, secreted fractalkine functions as a chemoattractant in recruiting T cells and monocytes to the sites of infection (1, 12, 18). In particular, fractalkine mediates the recruitment and activation of CX3CR1-expressing leukocytes including subsets of NK cells and CD4/CD8 T

lymphocytes to sites of infection (1, 12, 18). Interestingly, RSV G protein acts as a fractalkine receptor antagonist and functions to modulate host immune responses during RSV infection, particularly by modulating the fractalkine-mediated responses, including the alteration of pulmonary trafficking of CX3CR1-expressing immune cells and dysregulation of the timing and magnitude of cytokine and chemokine secretion (15, 25). Previously, mice challenged with a recombinant RSV strain lacking the entire G protein, or with a mutant RSV strain containing a point mutation at the CX3C motif, were show to have increased pulmonary trafficking of NK, CD4+, and CD8+ cells compared to mice challenged with wild type RSV (15). These results suggested that RSV G protein via its CX3C motif may interact with CX3CR1 to dysregulate immune cell trafficking to the site of infection, and such manipulation of the host immune response may contribute to enhanced disease pathogenesis. Importantly, mice vaccinated with polypeptides containing the G protein CX3C motif generated antibodies that inhibited G protein CX3C-CX3CR1 binding and chemotaxis, reduced lung virus titers, and prevented RSV infection-mediated body weight loss and pulmonary inflammation (29), suggesting a RSV vaccine that induces antibodies that block G protein CX3C-CX3CR1 interaction may prevent aberrant modulation of the immune and inflammatory responses to RSV infection.

These findings led us to our **central hypothesis** that modifications to the RSV G protein that eliminate the CX3CR1 binding region may improve vaccine safety while induction of antibodies to this region may block G protein CX3C-CX3CR1 interaction and improve vaccine efficacy. To address this hypothesis, we evaluated the regions in the 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 **specific hypothesis** is that vaccination with RSV G protein polypeptide spanning the central conserved

region can induce antibodies that block G protein CX3C-CX3CR1 interaction, preventing G protein-mediated immune modulation and offering cross-protection against both A and B subtypes of RSV. The **long-term goal** of our research is to determine 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 **rationale** for this approach is that successful completion would provide the scientific foundation for development of new RSV vaccine strategies to prevent RSV disease. The specific aims addressed are:

Specific Aim 1: To determine the regions within the RSV G protein that can induce antibodies against the central conserved region containing the CX3C chemokine motif.

Specific Aim 2: To determine the ability of polyclonal antibodies generated in mice immunized with the RSV A2 G protein polypeptides to inhibit RSV A2 and RSV B1 G protein CX3C motif binding to CX3CR1.

Specific Aim 3: To determine the ability of the RSV A2 G protein polypeptide-specific polyclonal antibodies that block the RSVA2 and RSVB1 G protein CX3C motif binding to CX3CR1 to cross-neutralize RSV A2 and RSV B1.

Nanoparticle and microparticle vaccines can be designed to mimic the structures of viral and bacterial pathogens. The pathogen-associated molecular patterns (PAMPs) on these subunit vaccines can activate pattern recognition receptors (PRRs) that include the Toll-like receptors (TLRs) and induce signaling pathways that drive robust immune responses against the nanoparticles or microparticles even in the absence of conventional adjuvant. One way of

creating nanoparticles/microparticles for vaccination is by using layer-by-layer (LbL) assembly of polypeptides. This technique involves a sequential overlaying of oppositely charged polypeptides onto an internal calcium carbonate core such that the oppositely charged polypeptides are deposited on top of each other via electrostatic interactions (6). Using the LbL assembly technique, nano-/microparticles can be engineered with varying characteristics due to the flexibility in size, shape, and composition of the internal core substrate as well as the choice of polypeptides that can be used to layer the nanoparticle. For example, LbL assembly can be used to produce artificial red blood cells (22), as well as controlled-release particles loaded with insulin or vitamins (5, 9). In addition, LbL can be utilized to produce virus-like particles expressing immunogenic epitopes to be used as a platform for vaccine delivery (21). Previous studies utilizing LbL nanoparticles as the vaccine delivery mechanism reported that vaccination with the layer-by-layer nanoparicles incorporated with well-defined antigenic epitopes improved both humoral and cell-mediated immune responses to OVA (10, 11, 27, 28), hepatitis B (19), tumor antigens (14, 24), and RSV (20) models.

LbL technology represents a novel approach in RSV subunit vaccine development and led us to our **Specific Hypothesis** that vaccination with RSV G polypeptide spanning the CX3C motif within the central conserved cysteine noose region can induce immune responses that are safe and effective in preventing RSV infection, and the optimal induction of such immune responses can be achieved via vaccination with LbL nanoncapsule containing the peptide sequences of the cysteine noose region. The **long-term goal** of this research is to develop a safe and effective nano-/microparticle RSV subunit vaccine engineered to express RSV G polypeptides that are able to induce protective immune responses against RSV infection, and our **rationale** is that successful completion would outline a novel strategy in the development of

RSV vaccines that are effective in conferring protective immunity against heterotypic RSV infections while preventing RSV G protein-mediated immune modulation and disease pathogenesis. It is believed that nano-/microparticle vaccines can be designed using LbL technology to produce synthetic subunit vaccines carrying target polypeptides comprising the CX3C motif from the G protein of RSV A and/or B strains. In this study, we test RSV G-polypeptide subunit vaccines that induce antibodies that induce neutralizing antibodies and that block G protein-CX3CR1 interaction as a novel strategy in the development of safe and effective RSV vaccines.

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CHAPTER 2

LITERATURE REVIEW

Respiratory Syncytial Virus (RSV)

Human respiratory syncytial virus (RSV) is an enveloped, negative-sense single-stranded RNA virus in the Paramyxovirus family, which was first isolated from a chimpanzee in 1956 and subsequently from two children in 1957 (22, 33). Since its isolation, RSV has become recognized as the single most important cause of serious lower respiratory tract disease in infants and young children, and of morbidity and some mortality in the elderly and immunocompromised (22, 33, 34, 48, 64, 69). Peak incidence of disease occurs in the first six months of life when maternal antibodies are present. Infection results in bronchiolitis, and, in severe cases, pneumonia, which often results in hospitalization (232). In addition, RSV infection is associated with secondary infections, such as otitis media, and can predispose young children for asthma-related illness later in life (109, 170, 184, 218). Unfortunately, natural infection provides only limited protective immunity and humans experience repeated infections and disease throughout life (69, 70, 184).

RSV possesses a 15.2kb genome which contains 10 genes encoding 11 different proteins (12, 59, 122, 183). The RSV genome is sequentially organized into two non-structural (NS2, NS1) genes followed by nucleocapsid (N); phosphoprotein (P); matrix (M); small hydrophobic (SH); attachment (G); fusion (F); second matrix (M2), which encodes two proteins from M2-1/M2-2 open reading frames (ORF) that have roles in RNA transcription and replication; and

RNA-dependent RNA polymerase (L) genes (3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5') (39, 44, 92, 115).

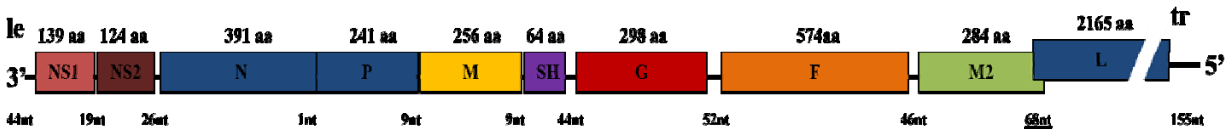

Figure 2.1. A schematic map of the RSV genome (RSV strain A2), approximately to scale. Numbers above the map denote the amino acid (aa) lengths of the each viral protein. Numbers below the map indicate the nucleotide (nt) lengths of the 3' leader (le), 5' trailer (tr), and intergenic regions. The nucleotide length of the gene overlap is underlined. The genes indicated in the figure are as follows: NS1, nonstructural protein 1; NS2, nonstructural protein 2; N, nucleoprotein; P, phosphoprotein; M, matrix protein; SH, small hydrophobic glycoprotein; G, attachment glycoprotein; F, fusion glycoprotein; M2 protein; L, large polymerase protein.

RSV virions are pleomorphic is size and shape (12, 122, 183). The lipid envelope, derived from the host cell plasma membrane obtained during the budding process, protects the nucleocapsid and anchors three virally encoded surface transmembrane proteins: F, G, and SH (12, 122, 183). The two major transmembrane proteins, G and F, are primarily responsible for virus attachment and fusion, respectively. The RSV G and F proteins are also responsible for inducing protective immunity against RSV. Of all surface glycoproteins, the F protein is most effective in generating protective immunity via inducing neutralizing antibodies (187). Similarly, the G protein induces some neutralizing antibodies and contributes to the generation of protective immunity. However, the G protein also serves an important role in the modification of host immunity, adversely affecting RSV disease pathogenesis (107, 253, 256).

RSV primarily infects respiratory epithelial cells by attaching to highly sulfated cell surface iduronic acid-containing glycosaminoglycans (GAGs) such as heparin, heparan sulfate, and chondroitin sulfate B (103). A recent study indicated that the RSV F protein may also play a role in viral attachment via interaction with nucleolin expressed on the host cell membrane (245). Following attachment, the F protein is activated mediating entry and fusion with the host cell membrane in a process thought to be similar to influenza hemagglutinin-mediated fusion (74, 75, 226). Although studies using mutant RSV lacking the G protein have shown that the G protein may be dispensible for viral infection, the G protein is required for efficient viral replication in vivo (123, 247, 250, 256). Following fusion, the viral nucleocapsid is internalized into the host cell cytoplasm where polymerase subsequently initiates viral transcription and replication. The resulting viral gene products and newly manufactured nucleocapsids localize and accumulate near the host cell plasma membrane where progeny virions are assembled and released.

RSV replication cycle

RSV replication initiates when G protein binds cell surface glycosaminoglycans (GAG) via its heparin-binding domains (148). Upon attachment, F protein insert its F1 domain into the cell membrane followed by a series of conformational changes that ultimately results in the fusion of viral and cell membranes (144, 280). Following fusion, the viral nucleocapsids and polymerase are internalized into the cell cytoplasm where the polymerase initiates viral transcription and replication. Transcription of the non-segmented viral genome consisting of ten genes encoding 11 proteins takes place in a 3' to 5' direction from a single promoter in the 3' leader sequence and proceeds sequentially with termination and re-initiation of polymerase activity at each of the gene junctions. The transcription results in a gradient expression of a

series of subgenomic mRNAs with the genes located closer to the 3' end being transcribed at a higher rate than the genes at the 5' end (43). Once sufficient viral proteins have been translated, a shift from transcription to replication, mediated by M2 protein, follows, where eventually, different viral proteins localize near the cell plasma membrane where progeny virus are assembled and released by budding (20). It has been observed that RSV budding occurs in the apical surface of polarized epithelial cells while such directional budding was not observed in non-polarized epithelial cells (27, 212). Also, F protein can mediate viral fusion into neighboring cells causing formation of syncytia.

RSV Nucleocapsid Associated Proteins - L, N, P Proteins

The large viral polymerase (L) protein is the least abundant of the structural proteins. The L protein serves as the viral RNA dependent RNA polymerase and carries out RNA synthesis. It also performs mRNA capping at the 5' end and polyadenylation at the 3' end (153, 237, 278). In comparison with other Paramyxovirus, the L protein is similar in length containing approximately 2,200 amino acids with limited sequence homology to polymerase proteins in other paramyxoviruses (237). The template used by the L protein during RNA synthesis is the viral ribonucleoprotein (RNP) or nucleocapsid, a complex of viral genomic and antigenomic RNAs tightly bound by N proteins (97, 243).

A crystal structure of the RSV N protein was recently revealed following its isolation as a decamer ring in association with bacterial RNA (243). This decamer ring likely surrounds one turn of the nucleocapsid helix as it has been reported that seven nucleotides of the viral RNA contacts each N protein subunit (243). This crystal structure also revealed an unique feature within the RSV N protein that sets it apart from the N proteins of other members of the order

Mononegavirales as the N-terminal domain of the RSV N protein contains a long β hairpin structure projecting away from the core that may serve as a site of contact with the L polymerase (243). Also, the C-terminus of the N-was shown to extend above the plane of the ring and may serve as the site of interaction with the P protein during RNA synthesis (243). The tight encapsidation of the viral genome and antigenome is a characteristic that all members of the Paramyxovirus family shares, and such a trait prevents degradation of the viral RNAs, which lack the stabilizing features of a 5′ cap and 3′ poly A tail (132, 264). It also is thought that such encapsidation is a defense mechanism against the detection of viral RNAs by pattern recognition receptors in the host cell such as the cytoplasmic helicases RIG-I and MDA-5, which detect cytoplasmic tri-phosphorylated RNA and dsRNA and initiate signal transduction to activate the cellular transcription factors IRF3 and NFκB involved in inducing type I interferon (IFN) and pro-inflammatory cytokines (275-277).

The phosphoprotein or P protein of RSV functions as a co-factor of the viral polymerase, L protein, that is essential for the polymerase activity. The P protein is a homotetramer that have been described to interact with N, L, and M2-1 proteins (11, 82, 129). Promoter clearance and chain elongation by the viral polymerase L protein during transcription appears to be dependent on the P protein as P and L protein interaction seems to stabilize the polymerase complex (51, 62). While the N-terminus of the polymerase L protein is tethered to the P protein, the C-terminus of the P protein interacts with the C-terminus of the N protein to open up the nucleocapsid structure in order to enable the L protein interaction with the nucleotide bases of the viral RNA (113, 227). Moreover, the P protein serves as a chaperone during N protein synthesis by interacting with the growing N protein peptides to ensure correct assembly and delivery to the growing viral RNA chain during genome replication (32, 50).

RSV Transcription/Replication Regulation Factors - M2 proteins

The RSV M2-1 and M2-2 proteins are encoded by two overlapping ORFs within a single M2 gene, and they function as RNA synthesis factors regulating viral transition into transcription and replication (20, 37, 73). First, the M2-1 protein is a homotetramer that has been shown to bind the P protein and viral RNA in a competitive manner, suggesting that the P protein interacts with soluble M2-1 protein and delivers it to the RNA template (251). Meanwhile, studies have also shown that viral transcription terminates prematurely in the absence of the M2-1 protein, suggesting that M2-1 protein serve as a transcription elongation factor for the viral polymerase complex (37, 73). The other RNA synthesis factor, the M2-2 protein, is a small 11 kDa protein that may mediate a regulatory "switch" from transcription to RNA replication (20, 38). Interestingly, mutations that ablated M2–2 gene expression attenuate virus growth in cell culture by 1000-fold *in vitro*, and a 10-fold reduction in the final yield of cultivated infectious virus has been observed (20).

RSV Maxtrix Protein - M protein

The RSV matrix (M) protein is a structural protein that lines the inner surface of the viral envelope. Based on crystal structure, the RSV M protein is a monomer that is organized into a horseshoe-shaped topology with the N-terminal and C-terminal domains joined by a short peptide linker (171). The outer surface of the M protein is largely positively charged and may mediate electrostatic interaction with the host plasma membrane and the viral nucleocapsid (171). Previous studies have described that M proteins can be detected in cytoplasmic inclusions during late infection in association with the N, P, and M2-1 proteins and that the M protein functions to inhibit viral transcription (86, 88, 161). Localization in cytoplasmic inclusions,

interaction with nucleocapsid components, and the role it plays in the inhibition of viral transcription indicate that the M protein is essential for efficient viral assembly and budding via bringing cytoplasmic nucleocapsids into association with RSV envelope proteins (87, 88).

RSV Fusion Protein - F protein

The RSV fusion (F) protein is an envelope-associated surface protein that mediates viral penetration into host cells as well as fusion between the infected cells (40). The F protein is a type I membrane glycoprotein of approximately 70 kDa that contains a cleaved N terminal signal sequence anchored in the membrane by a C terminal membrane anchor sequence allowing a large portion of the N terminal residues to be extracellular (9, 10, 158). The F protein is synthesized as a precursor protein (F0) in the endoplasmic reticulum consisting of F2 domain (amino acids 1-130), a cleavage site (amino acids 131-136) and F1 domain (amino acids 137-574). The F0 precursor is cleaved by a furin-like enzyme into disulfide linked F1 and F2 chains in the trans-Golgi compartment, and this cleavage process transforms the F0 precursor into a fusion competent form (90, 282). Following the cleavage, the F2 domain is modified further via N-linked glycosylation, and the F protein is expressed as an oligomer, (trimer or tetramer) on the surface of the virion (17, 29).

Interestingly, the RSV F protein can mediate the entire viral fusion process on its own while, in the other members of *Paramyxoviridae* family, the fusion process commonly requires cooperation between the fusion protein and cognate attachment proteins. Additionally, as with other members of *Paramyxoviridae* family, the RSV F protein is present in the virus particle in a metastable pre-fusion structure (145, 240). Following binding to the host cell, the F protein goes through a series of conformational changes. The F protein undergoing the fusion-driven

conformational change first becomes a pre-hairpin intermediate, in which the hydrophobic fusion peptide at the N-terminus of the F1 chain is inserted into the host cell membrane. Further structural change results in the assembly of a highly stable post-fusion structure characterized by the formation of a six-helix bundle consisting of two heptad repeats from each F protein monomer (280), and the free-energy released during six-helix bundle formation drives the membrane fusion process (215).

A recent study indicated that the RSV F protein may also play a role in viral attachment via interaction with nucleolin expressed on the host cell membrane (245). Although the G protein is still considered to be the major attachment protein of RSV and is essential for proper viral replication *in vivo*, (123, 246, 247, 250), both F and G proteins have been shown to bind to cell surface glycosaminoglycans (GAGs) via their heparin binding domain, a process that has been described as necessary for the efficient infection of cell lines by RSV (75, 247). Taken together, these observations suggest that efficient attachment by RSV *in vivo* depends on both F and G proteins, and may depend on two different binding events involving GAGs and nucleolin.

RSV Attachment Protein - G protein

On the basis of monoclonal antibody studies comparing recognition of RSV G protein, RSV has been classified into two major groups, i.e. A and B (7, 174). The RSV G protein is unique in its structure and functions among the attachment proteins expressed by other members of the *Paramyxoviridae* family (219). RSV G protein is a highly glycosylated type II glycoprotein that contains a short intracellular region (amino acids 1-37) and a hydrophobic transmembrane region (amino acids 38-66), which serves as a signal peptide and membrane anchor, at its N-terminus (219, 263). The amino acid residues continuing beyond the

transmembrane region make up the extracellular region extending to the C-terminus (269). Depending on the virus strain, the mRNA coding for RSV G protein is translated into a 32 kDa polypeptide precursor of amino acid length 292-299 (160, 239). At the endoplasmic reticulum (ER), the G protein precursor is co-translationally modified via the addition of high mannose N-linked sugars to produce a 45 kDa G protein intermediate. This intermediate form is then transported to the trans-Golgi compartment where further modification to the G protein intermediate continues with the addition of O-linked sugars, generating the mature form of RSV G protein with the molecular weight of approximately 90 kDa (42, 270). Furthermore, following RSV infection, the G protein is produced in two forms: a full-length, membrane-anchored polypeptide with a short cytoplasmic domain (G_m) and a secreted, soluble polypeptide (G_s) with the truncated transmembrane domain (111, 112, 213). Studies have reported that approximately 15% of the G glycoprotein synthesized in the infected cells is secreted as a soluble form lacking the cytoplasmic domain and part of the signal-anchor sequence (213). However, the soluble form of G protein retains other structural characteristics of the membrane bound form and is hypothesized to play an important role in evasion of host immune responses (111, 112). The extracellular region of the RSV G protein is highly glycosylated and is hypervariable from amino acids 67-147, but contains the evolutionarily conserved central region from amino acids 148-198 followed by another highly glycosylated and variable region from amino acid 199 to the C-terminus (42, 150, 269, 270). The central conserved region contains a CX3C chemokine motif (amino acids 182-186) composed of four evolutionarily conserved cysteine residues at amino acid positions 173, 176, 182, and 186 that form a structure called “cysteine noose” (42, 150, 269, 270). The CX3C chemokine motif within the RSV G protein have been shown to antagonize fractalkine (CX3CL1) functions by competitively binding to immune cells expressing the

fractalkine receptor, CX3CR1 (106, 255). Therefore, the sequence of the RSV G protein that is common to both G_m and G_s forms, and among different strains of RSV, is of the central conserved region containing the four cysteine residues (167). Interestingly, previous studies have shown that strain-specific antibody responses primarily recognize epitopes within the hypervariable C-terminal region of the RSV G protein (159). Moreover, the glycosylation pattern of the RSV G protein varies with the infected cell types indicating that the different glycosylation patterns on the RSV G proteins may be one of the mechanisms to evade the host immune response via the alteration of the G protein antigenic profile (30, 83, 84, 191, 192). Such findings underscore the importance of antibody responses to the central conserved region of the RSV G protein in generating cross-protection against both strains of RSV.

RSV Small Hydrophobic Protein - SH protein

The RSV small hydrophobic (SH) protein is a short transmembrane glycoprotein containing approximately 64 amino acids that is anchored into the viral envelope by a hydrophobic signal-anchor sequence near its N-terminus while its C-terminus is oriented extracellularly (41, 186). The SH protein exists in two different forms due to the use of two translational start sites during its synthesis and subsequent addition of N-linked sugars and olylactosaminoglycan (6, 186). When expressed in *Escherichia coli*, SH protein increases the bacterial membrane permeability toward ions and small molecules (197). Additionally, when the RSV SH protein was inserted onto artificial membranes, a formation of pore-like homo-oligomer structures that possess ion channel-like function was observed (31, 81). Interestingly, the SH protein shares structural similarity with viroporins, a class of hydrophobic protein that inserts itself into the host membrane and induces permeability to ions and small molecules (91).

Moreover, the SH protein may also play a role in modulation of the host immune response. The RSV SH protein was reported to have similar functions as that of the Parainfluenza 5 virus (PIV5) counterpart in regulating apoptosis of infected cells and inhibiting tumor necrosis factor (TNF)-α expression and signaling (79).

RSV Nonstructural Proteins - NS1 and NS2 proteins

The RSV nonstructural (NS1 and NS2) proteins have an important role in suppressing the cellular antiviral responses via interfering with the induction and signaling pathways of the type I IFN response. The NS1 and NS2 proteins are small protein consisting of 139 amino acids and 124 amino acids, respectively (45). There is limited sequence homology between the NS1 and NS2 proteins; however, both NS1 and NS2 share the same four C-terminal amino acids (45). The NS1 protein has been described to decrease the levels of TRAF3, an important signal transducer in type 1 IFN gene induction pathway, and IKKε, a protein kinase that specifically phosphorylates and activates IFN regulatory factor 3 (IRF3) (234, 241). The NS2 protein has been described to interact with RIG-I and antagonize the RIG-I mediated IFN gene promoter activation, resulting in the inhibition of type-1 IFN gene transcription (151, 234, 241). Importantly, the NS2 protein also is responsible for the decreased level of cellular TRAF3 and proteasomal degradation of STAT2, which serve as a signal transducer of activated IFN-α/β receptor in the JAK/STAT pathway that leads to transcriptional activation of the interferon stimulated response element (ISRE) genes (151, 207). Since type III IFN uses STAT1 and STAT2 as its downstream signal transducers, NS proteins can also suppress the induction of IFN-λ in human epithelial cells and macrophages (60, 233). Also, RSV NS proteins may possess an intrinsic anti-apoptotic property since they suppress tumor necrosis factor (TNF)-α mediated

apoptosis of infected cells during early infection and prolong their survival (21). Such properties of the RSV NS proteins can be viewed as a mechanism to increase the yield of the progeny virions.

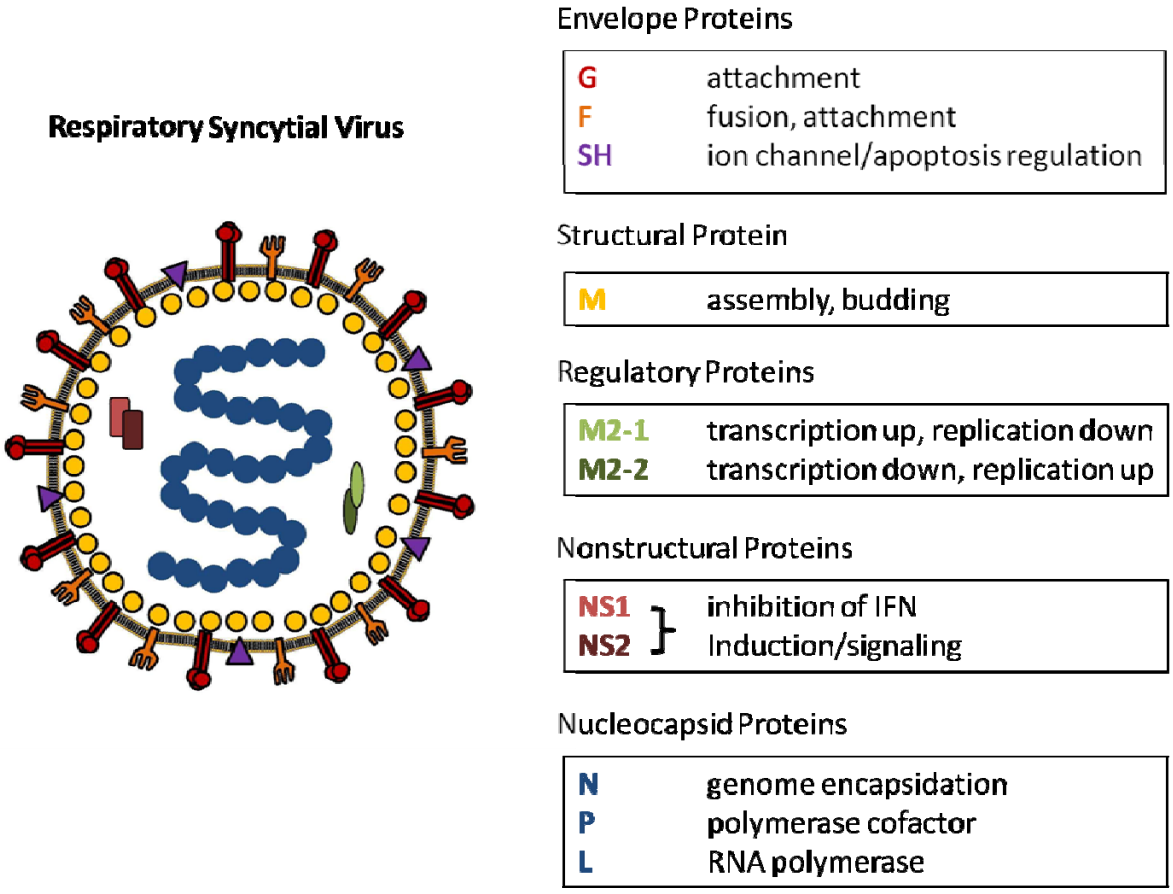

Figure 2.2. A schematic diagram of RSV virion (not to scale) and a summary of RSV protein functions.

Innate Immune Responses against RSV infection

Innate immunity is responsible for early host resistance to RSV infection, and it facilitates induction of adaptive immunity. Upon RSV infection, pattern recognition receptors (PRRs) expressed by the antigen presenting cells (APCs) and airway epithelial cells detect RSV

proteins and carbohydrates expressed during infection. There are various types of PRRs, and they can be either membrane-bound like Toll-like receptors (TLRs), mannose receptors, and scavenger receptors, or cytoplasmic like nucleotide oligomerization domain (NOD)-like receptors and retinoic acid inducible gene-I (RIG-I)-like receptors (125, 128, 130). TLRs expressed on the cell surface recognize extracellular bacterial and viral components while TLRs located endosomally recognize viral or bacterial nucleic acids that enter the endosomes by endocytosis (128, 130). Different TLRs are involved in sensing different pathogen types, and the engagement of the TLRs by their pathogen-associated-molecular patterns (PAMPs) initiates downstream innate immune signaling leading to the secretion of proinflammatory cytokines, especially type I IFNs, induction of the antiviral state, and subsequent recruitment of leukocytes to the site of infection. To date, RSV has been reported to interact with TLR2/6, TLR3, TLR4, and TLR7 (80, 98, 108, 141, 152, 179, 221, 225). As NOD-like receptors and RIG-I-like receptors survey the cytoplasm for replicating viral RNAs, they also have an important role in the recognition of RNA viruses, a feature that leads to the activation of innate immune responses including the induction of type I and III interferon (IFN) responses and secretion of other inflammatory cytokines to block viral replication and to facilitate immune activation (125, 128, 217).

Generally, TLR2 recognizes peptidoglycan on Gram-positive bacteria, and TLR4 recognizes lipopolysaccharide (LPS) on Gram-negative bacteria (128, 130). Interestingly, previous studies have reported that TLR2, TLR4 and TLR6 are involved in the recognition of RSV and activation of innate immune following RSV infection (100, 141, 179). Activation of these TLRs by RSV results in increased expression of TNF-α, IL-6, MCP-1 (CCL2), and RANTES (CCL5) (100, 179, 259, 273). Additionally, TLR2 and TLR6 signaling during RSV

infection has been linked to neutrophil recruitment and DC activation in the lungs, suggesting that TLR2 and TLR6 are involved in the activation of innate immune responses during RSV infection (179). RSV F protein has been reported to interact with CD14 and TLR4 (141), and TLR4 appears to be essential for induction of innate immune responses to RSV infection (108). For example, TLR4-deficient mice challenged with RSV exhibited impaired recruitment of NK cells and CD14+ cells to the lungs as well as deficiencies in NK cell-mediated killing, interleukin (IL)-12 production, and virus clearance (108). Furthermore, RSV-specific activation of TLRs on APCs and phagocytic cells can trigger adaptive immune responses by up-regulation of cytokines responsible for T lymphocyte activation, clonal expansion, and recruitment, playing a key role in bridging the innate and adaptive immune responses. TLR3, an intracellular PRR that recognize viral replication intermediates, also plays critical role in the regulation of immune environment in the lungs following RSV infection. Studies have revealed that RSV infection in TLR3 knock-out mice led to increased production of TH2 cytokines (IL-5 and IL-13) and mucus production in the lungs (214).

Dendritic cells (DCs) are professional antigen presenting cells (APCs) patrolling the local environment for invading pathogens. Using their phagocytic ability, DCs capture various antigens and present them to T cells as peptides in the context of either major histocompatibility complex (MHC) I or II (13). DCs interact with CD8+ T cells via MHC I leading to the activation and differentiation of the antigen-specific cytotoxic T lymphocytes (CTLs) (13). DCs also interact with CD4+ T cells via MHC II leading to the induction of helper T cell responses. In addition, DCs can detect the presence of invading pathogens via PRRs, and the recognition of PAMPs via PRRs induce DC maturation (13). DC maturation leads to an up-regulation of the antigen-specific MHC expression (13, 118). The expression of co-stimulatory molecules, such

as CD80 and CD86, are also up-regulated upon DC maturation in order to foster expansion of the pathogen-specific T cell population (13, 118). Moreover, mature DCs secrete cytokines such as IL-12 and type I and type II IFNs that mediate host defense against invading pathogens (13, 118). Interestingly, DC maturation can be negatively affected by RSV as the result of NS1 protein mediated type 1 IFN antagonism, leading to a skewing of T cell responses toward Th2 type (175, 176). Such interference with DC maturation results in decreased antigen presentation and inadequate T cell activation, ultimately leading to incomplete or weak RSV-specific immune responses contributing to the likelihood of future reinfection.

DCs are classified into two main types: myeloid or conventional DCs (cDC, CD11b+, CD11c+) primarily expressing IL-12 and plasmacytoid DCs (pDCs, CD11b-, B220+) primarily expressing IFNα (13, 118). Interestingly, a balance between cDC and pDC in the lung and the draining lymph node seems to be critical in the determination of the pulmonary disease outcome during RSV infection (228, 229). Previous studies have revealed that increased pDC levels have a protective impact on the overall immune environment while depletion of pDC from the lungs of RSV infected mice results in enhanced pulmonary disease characterized by increased Th2 cytokine response (228, 229). Moreover, DCs themselves can be infected by RSV, and RSV infected DCs can cause impaired T cell activation characterized by lower T cell proliferation and poor cytokine secretion (53). Furthermore, RSV infected DCs show down-regulated expression of the CCR7 chemokine receptor which is necessary for DC migration to the secondary lymphatic tissue (147).

Neutrophils are short-lived granular polymorphonuclear cells containing primary azurophilic granules and secondary granules. The primary azurophilic granules contain myeloperoxidase together with other anti-microbial agents including defensins and cathepsin G.

The secondary granules contain lactoferrin and cathelicidin. High levels of neutrophils infiltration in the lung tissues and airway lumen have been associated with RSV bronchiolitis, indicating that neutrophils and their products may play an important role in lung pathology during RSV infection (68, 266). Interestingly, previous studies have shown that RSV infection can stimulate airway epithelial cells *in vitro* to produce and secret IL-8 (76, 162, 182, 194). As IL-8 is a potent neutrophil chemoattractant, it is likely that IL-8 plays a central role in neutrophil influx into the airway seen in RSV infection (140).

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that play a critical role in the clearance of virus-infected cells during early infection and function at the interface between innate and adaptive immunity. There is an initial influx of NK cells to the lungs during early RSV infection with levels peaking at around day 3-4 post-infection (256). Beta-chemokines, such as MIP-1α, MIP-1β, RANTES, and MCP-1 are important for the recruitment of NK cells to the site of infection and in enhancing NK cell effector functions (242). Interestingly, the presence of alveolar macrophages seems to be essential for the proper induction of NK cell effector functions during RSV infection, as depletion of macrophages reduced the activation and recruitment of NK cells (204). Beside its cytotoxic functions, NK cells have been shown to interact with DCs and facilitate proper DC maturation. NK cells also produce IFNγ that is responsible for subsequent induction of adaptive immune responses by enhancing the activation and differentiation of CD4+ T cells into Th1 type cells and CD8+ T cells into cytotoxic T lymphocytes (CTL).

Macrophages are important players in the fight against various respiratory pathogens, serving multiple functions as one of the key effectors of the innate immune system. Previous studies indicate that alveolar macrophages serve as a significant source of pro-inflammatory

cytokines, such as TNF-α, IL-6 and IL-8, following RSV infection (16, 204). Alveolar macrophages are also major producers of type I IFN, suggesting their importance in the induction of early immune responses to RSV infection (139). Accordingly, in macrophage-depleted mice, RSV challenge significantly inhibited early NK cell recruitment coincident with enhanced the peak virus titers in the lungs, while showing no significant affects on T lymphocyte recruitment, weight loss, and overall lung function. These results suggest that macrophages may play an important role in mediating earlier immune responses against RSV infection but have a minor role in later immune responses (204). Furthermore, another study has shown that the deficiency of alveolar macrophages in NZB mice were central to enhanced disease. The authors in this study observed that the depletion of alveolar macrophages in BALB/c mice prior to RSV challenge resulted in airway occlusion and a similar pathology was observed in macrophage deficient NZB mice, suggesting that macrophages may play an important role in restricting the lung viral load and in clearing apoptotic cellular debris which otherwise can promote severe RSV-related lung disease (209).

Chemokine and Cytokine Responses during RSV infection

Chemokine secretion by RSV-infected epithelial cells are crucial for the recruitment of monocytes, neutrophils (via IL-8/CXCL8), T cells (via RANTES/CCL5), and eosinophils (via eotaxin-1/CCL11) to the site of infection. Previous *in vitro* studies have shown that RSV-infected respiratory epithelial cells secrete CC and CXC chemokines such as IL-8/CXCL8, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2, IP-10/CXCL10, eotaxin-1/CCL11, macrophage chemoattractant protein 1 (MCP-1), and RANTES/CCL5 (182, 188). Accordingly, increased expression of these chemokines has been shown in the nasal washes of RSV-infected mice and

humans (19, 49, 99, 223, 249). Immune cells recruited to the site of infection, in turn, release inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 as well as IL-10, a cytokine closely associated with the development of virus-induced acute asthma (95, 117, 165). Secretion of these pro-inflammatory chemokines and cytokines may contribute to airway inflammation, bronchial hyper-responsiveness, and exacerbated mucus production observed during RSV infection. Accordingly, mice treated with anti-RANTES antibodies showed overall reduction in lung disease upon RSV infection (248). In another study mice treated with anti-CCL11 showed reduced lung eosinophila and disease severity in association with a reduction in Th2 cytokine secretion and CD4+ T cell influx in the lungs (163). Similarly, RSV infected MIP-1α knock-out mice showed reduction in lung pathology compared to the wild-type mice (99). Taken together, these findings underscore the role of chemokines as important mediators of RSV-induced lung disease.

Upon RSV infection, epithelial cells and infiltrating leukocytes release type I IFNs to counter early viral replication. IFNα and IFNβ signal through the IFNAR receptor on the target cell surface (85, 236). Once the IFNAR receptor is engaged, the IFN-induced signal is transported via activation of the receptor-associated janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2) through tyrosine phosphorylation, which in turn stimulates tyrosine phosphorylation of STAT1 and STAT2. Consequently, binding of type I IFNs to their cognate receptors induces activation of interferon stimulated genes (ISGs), products of which interfere with viral replication and viral gene transcription (85, 236). Furthermore, ISG activation enhances NK cell functions (114).

It has been shown that type III IFNs also have important roles in the defense against respiratory viruses, including RSV (172). Type III IFNs consist of IFNλ1, IFNλ2, and IFNλ3

(135, 224). Unlike other IFN types, type III IFNs use a distinct receptor complex (IL10Rβ and IL28Rα) for signaling, which is expressed only on limited cell types, including respiratory epithelial cells (224). Although the two IFN types bind to different receptor complexes, both type I and III IFNs share the same post receptor signaling components. As a consequence, binding of type III IFNs to their receptor complexes triggers JAK/STAT signaling, which ultimately results in the activation of antiviral genes (61). As such, both type I and type III IFNs induce highly similar gene expression profiles, indicating that both IFN types serve similar functions (281). Interestingly, previous studies have reported that the absence of either IFNAR or IL28R does not increase susceptibility to RSV replication in mice, whereas STAT1 deficiency does (121, 172). However, in mice deficient in both IFNAR and IL28R, a dramatic increase in RSV replication was observed (172), suggesting that the presence of either functional IFN type maybe sufficient to resist RSV replication.

Humoral Immune Responses against RSV infection

Secreted antibodies in serum, lymph, and mucosa mediate humoral immunity. RSV infection induces antibody responses against several viral antigens, however, only the two viral membrane-associated F and G proteins are responsible for inducing neutralizing antibodies which have a major role in protection against RSV infection (46, 238). One study using recombinant vaccinia virus expressing individual RSV proteins demonstrated that serum antibodies can be induced against the RSV F, G, M2 and P proteins, but only F and G protein were the major inducers of protection (46). There are five different classes of antibodies or immunoglobulins (Ig): IgA, IgD, IgE, and IgG. The first line of antibody-mediated resistance to RSV infection in the upper airways is mediated by secretory IgA in the mucosal lining. IgA

serves important protective functions in the respiratory tract due to its abundant presence in the mucosal linings of the lungs. IgA is also present in saliva, tears, and digestive tract mucosal linings. In the respiratory tract, once secreted by plasma cells in subepithelial tissues, dimeric IgA linked by J chain binds poly-Ig receptor at the basolateral surface of mucosal epithelia, gets transported across the epithelial barrier to the lumen, and is released into the mucus covering the respiratory tract. Hence, secreted dimeric IgAs in the mucosa can bind glycoproteins of invading RSV virions and inhibit their attachment and entry into the respiratory epithelial cells. Once targeted and bound by the secreted IgAs, the invading RSV virions are sequestered in the mucus and cleared out by ciliated epithelial cells in the mucosa. Interestingly, the presence of neutralizing IgA in the respiratory mucosa may be essential in maintaining defense against future RSV infection, as a previous study on experimental RSV infection in healthy adult volunteers reported that the levels of mucosal IgA neutralizing antibody correlates more closely with protection compared to levels of serum antibodies (168).

Although IgA antibodies in the respiratory mucosa may offer protection against RSV infection, IgA-mediated protection is transient and incomplete. Durable humoral protection is provided by IgM and IgG antibodies that bind and neutralize RSV. During a primary RSV infection, IgM antibodies are secreted initially, followed by secretion of IgG antibodies after the activated B cells go through helper T cell-mediated isotype switching and affinity maturation. For example, vaccination with the RSV G protein polypeptides would expose the host to the immunogenic epitopes within the RSV G protein and induce a primary humoral response against those epitopes in the RSV G protein. In this circumstance, secreted antibodies would predominantly be composed of IgM antibodies followed by IgG antibodies at later time point. However, upon natural infection with RSV following the G protein polypeptide vaccination,

existing memory B cells remaining from the prior exposure to the immunogenic epitopes within the RSV G protein would rapidly be activated and predominantly secrete IgG antibodies in greater efficiency than during the primary humoral response against the RSV G protein. As the result of having undergone affinity maturation, IgG antibody possesses higher affinity for its target epitope compared to other immunoglobulin isotypes. Hence, the binding of IgG antibodies to their targets would be more efficient than other immunoglobulin isotypes, corresponding to IgG antibodies' better efficacy in neutralizing RSV. In addition, IgG is abundant in serum and circulated in blood, playing a role in defense against systemic RSV infection as RSV has been shown to infect neuronal cells and establish persistent infection (149). IgG may also have an important role in mucosal defense. A previous study has reported that transportation of IgG across the mucosal epithelial of the human and mouse lung is mediated by MHC class I-related Fc-receptor, FcRn (235). Also, transportation of serum IgG across the capillary walls into the genital mucosa via imbalance in hydrostatic pressure has been observed (222), and similar mechanisms of IgG transportation across the respiratory epithelia has been proposed to participate in protection against respiratory infections. Currently, the best preventative strategy for high-risk infants against RSV infection is to treat them with neutralizing IgG monoclonal antibody (67).

In infants, passively transferred maternal IgG antibodies provide some protection from infection. Studies have associated the presence of maternal serum antibodies in early infancy to infrequent occurrence of RSV infection in infants under 8 weeks of age (36, 47). However, the maternal antibody levels diminish during the first 6 months of life leaving infants unprotected against RSV infection (25). Although RSV-specific antibodies appear within days of a primary RSV infection (164, 267), these antibodies are significantly lower in quantity than those of older

subjects (25, 180). Also, the antibody titer wanes after several months following primary infection (267). Such short-lived RSV-specific antibodies in infants has been attributed to their immunological immaturity as well as the suppressive effects of maternally transferred antibodies still remaining in the infants (47). Although the serum antibody levels following primary RSV infection generally wane quickly during infancy, higher RSV-specific serum antibody levels are maintained over the years after repeated RSV infection (180, 265).

Another mechanism in which IgG antibodies mediate their antiviral effects is by aiding the clearance of RSV infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC) (127, 185). The end product of ADCC is NK cell-mediated killing of RSV infected cells. On their cell membrane, NK cells express CD16, an Fc receptor that recognizes the Fc portion of the IgG. Once this Fc receptor on NK cell binds RSV-glycoprotein specific IgG that is bound onto the RSV glycoprotein expressed on the cell membrane of the RSV infected cell, NK cell releases cytotoxic granules containing perforin, granzymes, and various lytic enzymes as well as tumor necrosis factor (TNF) resulting in the death of the infected target cell (127, 185). Also, RSV infected epithelial cells can activate the complement cascade, and IgA, IgM, and IgG antibodies are involved in the activation of the complement cascade via the classical pathway (65, 126, 230). For example, RSV G protein-specific IgA, IgM, or IgG antibodies binding their target epitope within the RSV G protein expressed on the plasma membrane of an infected cell can initiate the complement cascade when C1 binds to the Fc portion of bound antibodies (65, 126). Activation of the complement cascade results in formation of membrane attack complex (MAC) on the plasma membrane of the target cell and subsequent death of the cell due to the loss osmotic stability. Hence, complement is an important component of innate immunity as well as humoral immunity during RSV infection. However, complement activation is also associated

with enhanced RSV disease mediated by activation of C3a resulting in airway hyperreactivity (166, 200).

Cell-Mediated Immune Responses against RSV infection

While the antibody responses offer protection against RSV infection via neutralization, ADCC, and complement-mediated cytotoxicity, the cell-mediated responses are crucial for viral clearance. During early infection, NK cells are recruited to the lungs. Once recruited to the lungs, NK cells kill virus infected cells and function as a major producer of IFNγ (157). Secretion of IFNγ by NK cells in the lungs subsequently leads to the recruitment of CD4+ helper T cells and CD8+ cytotoxic T cells (CTLs) (157). IFNγ enhances the differentiation of CD4+ lymphocytes into Th1 subset that fosters the generation of pro-inflammatory cellular and humoral immune responses (28). IFNγ also enhances the differentiation and proliferation of CD8+ CTLs which are essential for the clearance of RSV in the lungs (28). In addition, absence of IFNγ during early RSV infection has been shown to be responsible for predominant Th2 type cytokine response and subsequent Th2-driven eosinophilia (116).

Humans with compromised T cell immunity, and mice depleted of CD4+ and CD8+ T cells show prolonged shedding of virus, underscoring the importance of T cell immunity in viral clearance (101). Various aspects of RSV-specific CD8+ CTL response and its impact in viral clearance have been extensively studied. During a primary RSV infection, the CTL responses have been observed to reach the maximal potency at 7-9 days post infection, and the RSV-specific CD8+ CTLs could be found in the lungs and nasal turbinate of the RSV infected mice (5, 14, 138). During secondary infection, RSV-specific CD8 T cells could be found in the lungs as well as in the peripheral blood indicating that T cell responses become more systemic upon re-

infection (131). It has also been shown that subsets of CD8+ CTLs can redistribute themselves rapidly from the peripheral blood to the bronchoalveolar lavage (BAL) within few days of re-infection (131). Given that clearance of RSV closely correlates with the increase of the RSV-specific CD8+ CTL activity in the lungs (244), rapid mobilization of CD8+ CTLs to the lungs reflects the increased efficiency in viral clearance by the host during a secondary RSV infection. Moreover, in humans, RSV-specific CD8+ CTLs have been described to recognize epitopes within the F, M, M2 and NS2 proteins (35). Also, a study that investigated RSV specific CTL epitopes in BALB/c mice revealed that M2 protein contains an immunodominant H-2Kd-restricted epitope that is necessary for the induction of CTL responses (137), and a H-2Db-restricted CTL epitope in RSV M protein was identified in C57B1/6 mice (216). These discoveries have provided useful insight to the study of RSV immunodominance and the kinetics of RSV-specific CTL responses.

CD4+ helper T cell responses also play an important role in terminating RSV infection. During RSV infection, CD4+ T cells can differentiate into Th1 or Th2 type and secrete cytokines that enhances either cell-mediated immunity or humoral immunity. It is important to note that a previous study using recombinant vaccinia virus expressing RSV F or G protein demonstrated that the F and G proteins can prime for functionally distinct subsets of T cells (3). In BALB/c mice, it was observed that the F protein primes both CD8+ and CD4+ T cells toward Th1-type cytokine responses while the G protein primes CD4+ T cells toward Th2-type cytokine responses (3, 120). Activation of different subsets of CD4+ T cells influences the predominance of either Th1- or Th2-type responses by controlling the cytokine environment in the lungs. Hence, the priming of different subsets of CD4+ T cells contributes greatly to the maintenance of balance between the CD8+ CTL-mediated cellular responses and the B-cell-mediated humoral responses

during RSV infection and can potentially have a major influence in subsequent disease pathogenesis.

Furthermore, there is evidence indicating that RSV possesses CD4+ T cell epitopes in the central non-glycosylated region of the G protein. A previous study reported that CD4+ T cell epitopes in the non-glycosylated ectodomain of the G protein can be recognized by Th2 CD4+ T cells in mice (105). Other studies have reported that an immunodominant epitope within the central conserved region of the G protein can be recognized by both Th1 and Th2 CD4+ T cells in humans (54, 56). Importantly, it has been reported that adoptive transfer of T cells recognizing the G protein into RSV infected mice induces lung disease, characterized by pulmonary hemorrhage, neutrophil recruitment and eosinophilia, compared to the transfer of M2- or F protein-specific T cells (2). Since G protein-specific T cells were exclusively CD4+ cells with Th2 characteristics, it is likely that G protein-specific T cells contribute to the enhancement of disease severity via secretion of Th2 type cytokines, IL-4 and IL-5, further skewing the Th1/Th2 balance toward Th2 (2). Such findings emphasize the critical role that CD4+ T cells play in RSV immunity and disease pathogenesis.

RSV Disease Pathogenesis

RSV disease can manifest in various ways among different individuals. Symptoms of RSV infection can be fever, otitis media, upper respiratory infection, lower respiratory infection, bronchiolitis, and pneumonia. In some cases, abnormal respiratory functions can persist for a long time after the clearance of RSV infection. Both host and viral factors have been observed to contribute to RSV-induced disease. However, their exact roles and relative contributions to disease pathogenesis are still being debated.

A general consensus is that RSV replication is positively associated with disease severity. In accordance with this notion, a study was performed on healthy human volunteers infected with RSV A strain showed that RSV viral load correlates with the severity of disease (58). Similarly, another study by the same group revealed that the viral load in 3 month-old infants correlated with the severity of RSV disease as well (57). Meanwhile, several *in vitro* studies have demonstrated that RSV is not intrinsically a cytopathic virus with infection limited to the ciliated airway epithelial cells supporting the notion that RSV disease may be mediated by the host immunity (257, 271, 279). Accordingly, in a study performed using the cotton-rat model, reduction of lung virus titers via palivizumab treatment (a monoclonal antibody against the RSV F protein) had little effect on lung pathology while anti-inflammatory glucocorticosteroid treatment reduced lung pathology (205). In humans, however, neither reduction of virus replication via antiviral therapeutic treatment nor treatment with corticosteroids to counter inflammation significantly decreased RSV-induced lung disease (136, 143, 156).

It is well established that both CD4+ and CD8+ lymphocytes are important for terminating RSV replication in primary RSV infection of mice. However, CD4+ and CD8+ T cells have been shown to contribute to RSV disease as well, with CD8+ T cells playing more important role as a mediator of disease as the depletion of CD8+ T cells in mice resulted in modest disease severity and early recovery (93). A second study demonstrated that CD8+ CTL activity was optimal in the lungs of RSV-infected mice between 7 and 9 days post-infection, corresponding to the peak of RSV disease following primary infection in mice (4). Further, a predominant Th2 CD4+ T cell response during RSV infection was positively linked to the severity of disease. Th2-biased T cell responses following RSV infection can be manifest in symptoms such as mucus over-production, airway obstruction, wheezing, and pulmonary

eosinophilia. It has been suggested that infants are more prone to suffer from severe RSV disease due to their Th2-biased immunity remaining from their neonatal period (1). Further, increased IL-4 production due to a polymorphism in the *il4* gene has been observed to correlate with severe RSV lung disease as IL-4 is one of the cytokines that drives the differentiation of CD4+ T cells into Th2 cells (169). Moreover, a previous study has observed that infants who suffered RSV-induced brochiolitis or asthma were associated with increased RSV-specific IgE and histamine levels in their nasopharyngeal secretions, which can be associated with increased Th2 response (268).

Evasion of the Host Immune Responses

An example of RSV-mediated immune evasion is given by the function of the RSV nonstructural (NS) proteins in suppression of type 1 IFN responses during infection (154, 207, 208, 220, 233, 234). RSV NS2 protein has been identified as the type I IFN antagonist linked to specific downregulation of signal transducer and activator of transcription (STAT)-2 while NS1 protein, containing elongin C and cullin 2 binding sequences, has been shown to act as an ubiquitin E3 ligase to target STAT2 to the proteasome (66, 208). Likewise, bovine RSV NS proteins have also been shown to antagonize type I IFN response by inhibiting the activation of IRF3, a member of the interferon regulatory transcription factor family (23). Furthermore, RSV NS proteins have been reported to suppress premature apoptosis, prolonging the life of the host cell in order to promote better viral replication and growth (21). For both human and bovine RSV, NS antagonism of the antiviral response coincides with expression of RSV proteins that occur as early as five hours post-infection and may ultimately result in inhibition of the cytotoxic T lymphocyte (CTL) response (21, 134). Furthermore, the RSV F protein can interact with

TLR4 and CD14 in human monocytes leading to activation of nuclear factor (NF)-κB and production of proinflammatory cytokines TNFα, interleukin (IL)-6, and IL-12 (141). Interestingly, studies have found that RSV F protein interaction with TLR4 further contributes to immune evasion via modification of cytokine and chemokine responses by induction of suppressor of cytokine signaling (SOCS) proteins suggesting that this may be an important immune-evasion mechanism to reduce type I IFN expression to foster virus replication (85). RSV has also been shown to induce cyclooxygenase (COX)-2 (211). COXs are rate-limiting enzymes that convert arachidonic acid to prostanoids, and COX-2 is upregulated during inflammatory processes. Interestingly, TLR4 stimulation also results in upregulation of genes involved the inflammatory response including COX-2 suggesting the possibility of RSV F protein-mediated induction of COX-2 via TLR4 signaling (195, 210). Moreover, evidence shows that RSV G protein may also suppress TLR4-mediated cytokine production by interfering with NFκB activation leading to decreased proinflammatory cytokine production (198).

Another role of the RSV G protein in the evasion of host immune response is its ability to mimic immune proteins (8, 106, 146, 198, 255). For example, the central conserved region containing the four cysteine residues within the RSV G protein is homologous to a part of the TNF receptor and has been shown to modulate the innate immune responses during RSV infection (146). Since TNF-α and TNF-β are proinflammatory cytokines attributed to a large range of inflammatory conditions, it is possible that the RSV G protein may bind to TNF-α and TNF-β modulating the host antiviral responses during RSV infection (24). Interestingly, infection of mice with a mutant RSV lacking the G and SH genes resulted in higher numbers of natural killer (NK) cells recruited to the lung as well as increased IFNγ and TNFα production suggesting that the G and/or SH surface proteins inhibit NK cell recruitment and

proinflammatory cytokine production (256). Furthermore, studies have found that the G and/or SH proteins inhibit the expression of several important chemokines (such as macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and monocyte chemoattractant protein (MCP)-1, that attract NK cells to the lungs in mice (254). Additionally, the central conserved region contains a CX3C chemokine motif at amino acid positions 182–186, which is used to binds to CX3CR1, the receptor for CX3CL1 fractalkine (255). The CX3CL1 fractalkine functions in the recruitment of CX3CR1-expressing leukocytes, such as subsets of NK cells, $CD4^+$ T cells, and $CD8^+$ T cells, to the sites of infection (106, 107, 254). CX3CR1 mimicry by the G protein has been shown to facilitate RSV infection and alter CX3CL1 chemotaxis of human and mouse leukocytes to the lungs (8, 106, 107, 198, 255). Together, these findings suggest that RSV G protein functions to modulate both the innate and adaptive immune responses of the host during infection and adversely contributes to the disease pathogenesis by facilitating the evasion of host immune responses, rendering the interaction of the G protein CX3C motif with CX3CR1 an important target for RSV disease intervention strategy.

RSV Vaccine Development

Although substantial efforts have been invested in order to produce safe and effective RSV vaccines, none has yet been successful owing mainly to the difficulties in achieving correct balance of safety and efficacy. Formalin-inactivated RSV (FI-RSV) was the first RSV candidate vaccine. However, FI-RSV vaccine induced a poor serum neutralizing antibody response and caused enhanced respiratory disease characterized by pulmonary eosinophilia and predominant Th2 type cytokine responses following subsequent RSV infection (94, 181, 200, 252).

Live-attenuated RSV vaccine candidates also have experienced difficulty achieving an appropriate, balanced level of attenuation given that the RSV vaccine virus replication tends to be directly correlated with induction of immune responses, clinical symptoms, and immunogenicity (124, 199, 272). However, development of subunit vaccines against RSV infection appears to be more promising. Several RSV subunit vaccine formulations have been examined including purified F protein (PFP) vaccines (15, 18, 71, 96, 133, 177) and those targeting G protein. Evidence provided by previous studies indicates that the RSV F protein is important in inducing protective immunity (96, 187). Accordingly, subunit vaccines based on the RSV F protein isolated from infected cell cultures have been extensively evaluated in humans of various age groups (18, 71, 96, 177, 193, 258). Although the F protein-based vaccine candidates were well-tolerated by the human subjects, they were not sufficiently immunogenic (18, 71, 96, 177, 193, 258). Meanwhile, a subunit vaccine candidate BBG2Na has been shown to elicit immune response in small and large animals (26, 55, 203) and has been evaluated in human clinical trials. BBG2Na is a fusion protein, synthesized in bacteria, consisting of the central conserved region of the G protein from RSV A Long strain (amino acid sequence 130-230) fused to the C-terminus of an albumin-binding region of the streptococcal G protein (26, 55, 203). The phase I trials, performed with 108 healthy young adults, proved that BBG2Na is both immunogenic and protective (202). However, the vaccine studies were discontinued shortly after due to some unexpected development of type III hypersensitivity (purpura) in two healthy young adults during phase II trials (63, 199). Moreover, a recent study using virus-like particles (VLPs) demonstrated that mice immunized with VLPs carrying RSV F or G protein produced higher viral neutralizing antibody levels and exhibited significantly decreased lung virus titer following live RSV A2 challenge. Interestingly, this study demonstrated that RSV-G VLPs vaccination

conferred better protection against RSV challenge compared to RSV-F VLPs vaccination as determined by the reduction in lung virus titers and morbidity post-challenge (206).

Although the specific reasons for the lack of success in RSV vaccine development remain to be answered, several challenges that impede RSV vaccine development are well known. First, natural RSV infection does not fully protect from re-infection and provides only partial protection from the disease, suggesting it will be difficult to induce a protective immune response (89, 102, 110, 201). Second, antigenic and genetic differences among circulating RSV strains are likely sufficient to affect the level of cross protection induced by viruses from different groups (7, 119, 174, 196). Lastly, the most paramount challenge that encounters the development of new safe and effective RSV vaccine seems to be the tackling of RSV-mediated circumvention of host immunity.

Additionally, of 11 viral proteins encoded by the RSV genome, the two surface glycoproteins, F and G proteins, induce neutralizing antibodies and confer protective immunity (46, 238). Moreover, studies evaluating a BBG2Na vaccine candidate in combination with different adjuvants and by different routes of administration have shown a role for the RSV G protein in protection against RSV in small and large animals (26, 55, 203). Additionally, study that analyzed the serum immunogenicity against various RSV G epitopes using sera from RSV A strain- and RSV B strain-infected human subjects reported a significant increase in homo- and heterosubtypic IgG response against the central conserved region of the RSV G protein (178). Taken together the findings from these studies suggest the possibility that antibodies specific to the central conserved region of the RSV G protein may be able to cross-neutralized both A and B strains of RSV and provide cross protection.

Nanoparticle as RSV vaccine delivery mechanism

Particulate vaccines have been shown to induce potent immune responses in the absence of conventional adjuvants due to the recognition by immune cells of the particle structures, which mimic natural pathogens such as viruses and bacteria. By incorporating well-defined antigenic epitopes in micro- and nanoparticle constructs, investigators have demonstrated improved immunogenicity of both B and T cell epitopes in a number of model systems. Utilizing synthetic nanoparticles as a mechanism to deliver antigenic peptides for immunization is an attractive strategy to develop novel vaccines against various infectious diseases. Nanoparticle vaccines can be designed to closely mimic the structures of viral and bacterial pathogens. Immune cells can thereby recognized this mimicry of particle structures and induce robust immune responses against the nanoparticles even in the absence of conventional adjuvant. One way of creating nanoparticles for vaccination is layer-by-layer (LbL) assembly of polypeptides. This technique involves a sequential overlaying of oppositely charged polypeptides onto an internal core, such that the oppositely charged polypeptides are deposited on the top of each other via electrostatic interactions. Using LbL assembly technique, nanoparticles with various functions and characteristics can be engineered due to the flexibility in size, shape, and composition of the internal core substrate as well as the choice of polypeptides that can be used to layer the nanoparticle. For example, LbL assembly technique can be used to produce artificial red blood cells (190), controlled-release particles loaded with insulin and vitamins (52, 72). Meanwhile, this technique can also be utilized to produce virus-like particles expressing immunogenic epitopes to be used as a platform for vaccine delivery (189). Previous studies that have investigated the possible utilization of LbL nanoparticles as the vaccine delivery mechanism reported that vaccination with the layer-by-layer nanoparicles

incorporated with well-defined antigenic epitopes improved both humoral and cell-mediated immune responses in OVA (77, 78, 260, 261), hepatitis B (155), tumor (104, 231), and RSV (173) models. Such robust immunogenicity generated by the nanoparticle vaccination is attributed to more efficient stimulation of innate immunity (142, 262, 274). Previous studies have observed increased efficiency in phagocytosis, cross-presentation, and activation of dendritic cells (DCs) measured by cytokine production and costimulatory marker expression following LbL nanoparticle vaccination (142, 262, 274). With its flexibility in design which allows the manipulation of particle size and the contents of the incorporated antigenic peptides to engineer the construct that generate the desired immune responses, LbL nanoparticle technology is a promising candidate for the production of delivery mechanism for future RSV subunit vaccines.

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CHAPTER 3

ANTIBODIES TO THE CENTRAL CONSERVED REGION OF RESPIRATORY SYNCYTIAL VIRUS (RSV) G PROTEIN BLOCK RSV G PROTEIN CX3C-CX3CR1 BINDING AND CROSS-NEUTRALIZE RSV A AND B STRAINS

Youngjoo Choi, Caleb S. Mason, Les P. Jones, Jackelyn Crabtree, Patricia A. Jorquera, and Ralph A. Tripp. Accepted in Viral Immunology

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Abstract

Respiratory syncytial virus (RSV) is a primary cause of severe lower respiratory tract disease in infants, young children, and the elderly worldwide, and despite decades of effort, there remains no safe and effective vaccine. RSV modifies the host immune response during infection by CX3C chemokine mimicry adversely affecting pulmonary leukocyte chemotaxis and $CX3CR1^{+}$ RSV-specific T cell responses. This study investigated whether immunization of mice with RSV G protein polypeptides from strain A2 could induce antibodies that block G protein–CX3CR1 interaction of both RSV A and B strains. The results show that mice immunized with RSV A2 G polypeptides generate antibodies that block binding of RSV A2 and B1 native G proteins to CX3CR1, and that these antibodies effectively cross-neutralize both A and B strains of RSV. These findings suggest that vaccines that induce RSV G protein-CX3CR1 blocking antibodies may provide a disease intervention strategy in the efforts to develop safe and efficacious RSV vaccines.

Introduction

Human respiratory syncytial virus (RSV) is a ubiquitous negative sense, single-stranded RNA (ssRNA) virus in the Paramyxovirus family that causes serious lower respiratory tract disease in infants, elderly, and the immune compromised (26, 39, 41, 55, 75, 82). For decades substantial efforts have been made toward producing a safe and effective vaccine; however, none have been successful owing to the difficulties in achieving correct balance of safety and efficacy. Formalin-inactivated RSV (FI-RSV) was one of the first RSV candidate vaccines. However, FI-RSV offered poor protection and led to an enhanced disease characterized by pulmonary eosinophilia upon subsequent natural RSV infection (110, 203, 230, 288). Live-attenuated RSV vaccine candidates also have experienced difficulty in achieving an appropriate level of attenuation given that the replication of the vaccine virus tend to be directly correlated with the induction of immune responses, clinical symptoms, and immunogenicity (145, 229, 315). Given these findings, the development of subunit vaccines against RSV appears more promising. Several RSV subunit vaccine formulations have been examined including purified F protein (PFP) vaccines (18, 22, 84, 112, 154, 198) and those targeting G protein. For example, BBG2Na is a G protein subunit vaccine candidate that was shown to elicit robust immune responses in small and large animals (30, 63, 234), and had been evaluated in human clinical trials. BBG2Na is a fusion protein, synthesized in bacteria, consisting of the central conserved region of the G protein from RSV A Long strain (amino acid sequence 130-230) annealed to the C-terminus of an albumin-binding region of the streptococcal G protein (30, 63, 234). The phase I trials, performed with 108 healthy young adults proved that BBG2Na is both immunogenic and protective (233). However, the vaccine studies discontinued shortly after due to unexpected

development of type III hypersensitivity (purpura) in two healthy young adults during phase II trials (73, 229).

The explanations for the lack of success in RSV vaccine development are often specific to the vaccine type studied; however, shared challenges also impede RSV vaccine development. First, natural RSV infection does not fully protect from re-infection and provides only partial protection from the disease, suggesting it will be difficult to induce a protective immune response (103, 118, 130, 232). Second, antigenic and genetic differences among circulating RSV strains are likely sufficient to affect the level of cross protection induced by viruses from different groups (8, 140, 195, 226). Lastly, the RSV proteins have been shown to circumvent several areas of host immunity (9, 28, 123, 166, 228, 290-292).

RSV has been classified into two major groups, A and B, with primary differences between the two groups observed in the G protein (8, 195). The RSV G protein was first recognized as an attachment protein that allows binding to the host cell surface through glycosaminoglycans and heparin-binding domains on the G protein (168, 312, 313). Following RSV infection, the G protein is produced in two forms: a full-length, membrane-anchored polypeptide with a short cytoplasmic domain (G_m) and a secreted, soluble polypeptide (G_s) with the truncated transmembrane domain (131, 132, 245). The sequence of G protein that is common to both G_m and G_s forms of the protein consists of a conserved central region containing four cysteine residues and two variable mucin-like regions flanking the central conserved region (187). Studies have shown that strain-specific antibody responses primarily recognize epitopes within the hypervariable C-terminal region of the RSV G protein (178). Interestingly, the glycosylation pattern of the RSV G protein varies with the infected cell type indicating that the different glycosylation patterns on the RSV G proteins may be one of the mechanisms to evade

the host immune response via the alteration of the G protein antigenic profile (34, 97, 98, 221, 222). This underscores the importance of the antibody response specific to the central conserved region of the RSV G protein in generating cross-protection against both strains of RSV.

RSV G protein has an important role in modifying the innate and adaptive immune response. Several studies have shown that the chemokine, cytokine, and T cell response is affected by G protein expression (123, 290, 292, 294). CX3C chemokine mimicry mediated by the G protein (9, 123, 166, 228, 291) is attributed to the central conserved region which contains four cysteine residues (291). The G protein central conserved region also has structural homology with the fourth subdomain of 55-kDa tumor necrosis factor receptor (30). Since TNFα and TNFβ are proinflammatory cytokines affecting the inflammatory response, it is possible that the RSV G protein may bind to TNF-α and/or TNF-β thereby modulating their antiviral activities during RSV infection (28). Interestingly, infection of mice with a mutant RSV lacking the G and SH genes resulted in higher numbers of natural killer (NK) cells recruited to the lung as well as increased IFNγ and TNFα production suggesting that the G and/or SH surface proteins inhibit NK cell recruitment and proinflammatory cytokine production (292). Furthermore, studies have found that the G and/or SH proteins inhibit the expression of macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and monocyte chemoattractant protein (MCP)-1 that attract NK cells to the lungs in mice (290), and G protein has a CX3C chemokine motif at amino acid positions 182–186 which can bind to the fractalkine receptor, CX3CR1 (291). Fractalkine, CX3CL1, functions in the recruitment of CX3CR1-expressing leukocytes, such as subsets of NK cells, $CD4^+$ T cells, and $CD8^+$ T cells, to the sites of infection (123, 126, 290). CX3CR1 mimicry by the G protein has been shown to facilitate RSV infection and alter CX3CL1 chemotaxis of human and mouse leukocytes to the lungs (9, 123, 126, 228, 291). Together these findings

suggest that RSV G protein functions to modulate immunity and facilitate virus replication. Thus, disease intervention strategies that interfere with G protein-CX3CR1 interaction are potentially effective disease intervention strategies.

Although RSV G protein modulates immunity, it also can confer protective immunity similar to the F, or fusion protein (53, 271). Studies evaluating the BBG2Na vaccine candidate showed a clear role for the RSV G protein in protection against RSV disease in animals (30, 63, 234). Additionally, a study which analyzed serum immunogenicity against RSV G protein epitopes using sera from RSV A and RSV B infected human subjects reported a significant increase in homo- and heterosubtypic IgG response against the central conserved region of the RSV G protein (199). The findings from these studies suggest that antibodies specific to the central conserved region of the RSV G protein may be able to cross-neutralized both A and B strains of RSV and provide cross protection.

In the present study, we examined a RSV vaccine strategy using RSV G polypeptide vaccination to generate antibodies specific to the central conserved region of the G protein that block G protein-CX3CR1 interaction of RSV strain A and B viruses and provide heterosubtypic neutralization of RSV strain A and B. It is interesting to note that while RSV A2 G protein-CX3CR1 interaction and its functions associated with the RSV disease have been by described by previous studies, RSV B1 G protein-CX3CR1 interaction and the role it has during RSV infection has not been well documented. Our results show that antibodies specific to the central conserved region of the RSV G protein block binding of RSV A2 and RSV B1 native G protein to CX3CR1 and have some ability to cross-neutralize the two strains of RSV. A better understanding of the host humoral immune response associated with RSV infection is critical for development of safe and efficacious RSV vaccines and therapeutic treatments. This study

provides a vaccination strategy to prevent or reduce RSV G protein-mediated immune evasion and disease pathogenesis.

Materials and Methods

RSV G polypeptides for immunization

RSV G polypeptides spanning different regions of the RSV G protein were designed for vaccination (Figure 1). G polypeptides were commercially synthesized by Liquid Phase Peptide Synthesis and received as a lyophilized powder (GenScript, Piscataway, NJ). The peptides were resuspended to a stock concentration of 3 mg/ml with DMSO (Sigma, St. Louis, MO) and stored at 4C. For the studies, the peptides were diluted with PBS, aliquoted at 1 mg/ml, and held at 4C for general usage. For vaccination studies, PBS was used to dilute the polypeptides to the desired concentration. The G polypeptides that were used for immunization correspond to N-terminal variable, glycosylated region (G1 polypeptide, aa 67-147), the central conserved region (G2 polypeptide, aa 148-198), and the C-terminal hypervariable region (G3 polypeptide, aa 199-298) of the G protein of RSV A2 strain.

RSV native G protein purification

Native G protein was purified from RSV A2 or RSV B1 infected Vero E6 cells (MOI = 1) using affinity chromatography as previously described (Supplementary Figure 1); (291). Briefly, at day 5 post infection (pi), Vero E6 cells infected with RSV A2 or B1 were collected, 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 to 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 iterations, resting for 5 minutes in between cycles, followed by centrifugation at 10,000 x 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 monoclonal antibody (MAb 131-2G) according to the manufacturer's protocol. The column was equilibrated with 2 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 central conserved region of G protein of RSV. Lysate was loaded at a flow rate of 1 ml/minute. The column was washed with 4 volumes of PBS + 0.2% N-Octyl-β-glycoside and eluted with 4 volumes of 0.1M glycine, 1% N-Octyl-β-glycoside, pH 2.2, collecting 10 fractions of 2 ml. 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 method yields highly purified G protein with no detectable F protein by Western blot analysis and no detergent after dialyzing.

G polypeptide vaccination

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 of Georgia Institutional Animal Care and Use Committee. All RSV G polypeptides and UV-inactivated RSV A2 were emulsified in 1:1 ratio with TiterMax (Sigma) and mice were immunized intramuscularly (i.m.) with the total of 50 μg vaccine/mouse in the hind quarters. Each mouse was immunized with

100 µl of RSV G polypeptide + Titermax mixture (50 µl per hindleg). At day 14 post-vaccination, mice were boosted with equal amount of RSV G polypeptide + TiterMax or UV-inactivated RSV A2 + TiterMax emulsification. After receiving the boost, vaccinated mice generated RSV G protein-reactive antibody titer of > 3 standard deviations (SD) above the background determined by enzyme-linked immunosorbent assay (ELISA). The sera from the G polypeptide-vaccinated and UV-inactivated RSV A2-vaccinated mice were collected and stored at -80°C for further experiments.

ELISA

The antibody titers in sera collected from vaccinated mice and controls were determined using a modified indirect ELISA (322). Briefly, flat-bottom microtiter plates (Corning, Corning, NY) were coated with 1 µg/well of immunizing antigen, RSV A2 native G protein, or RSV B1 native G protein, overnight at 4°C. Serial dilutions of sera in PBS were added to the wells and incubated for 1 h at 37°C. The plates were washed three times with washing buffer (PBS containing 0.05% Tween) and incubated for 1 h at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Millipore, Temecula, CA). After being washed, the plates were developed with pNpp substrate (Pierce) as indicated by the manufacturer.

Transfection and selection of 293-CX3CR1 cells

Human 293 cells (CRL-1573; ATCC) were transfected with pcDNA3.1 expression plasmids (Invitrogen Corp., Carlsbad, CA) encoding CX3CR1 as previously described (291). Briefly, plasmid inserts were derived from genomic DNA by high-fidelity PCR amplification (Invitrogen) and were sequenced bidirectionally. After G418 selection for at least 3 weeks, stable

receptor expression was verified by flow cytometry. Stably transfected cells (293-CX3CR1) were stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CX3CR1 monoclonal antibody (MAb 2A9) obtained from MBL International (Nagoya, Japan). Cell sorting was performed using a DakoCytomation MoFLo high-speed cell sorter after gating of dead cells by use of propidium iodide and correction of results for nonspecific staining by use of isotype antibody controls. The expression level of CX3CR1 was determined by flow cytometry and showed that >85% of 293-CX3CR1 cells expressed CX3CR1 compared to the untransfected 293 cells.

G protein-CX3CR1 Binding Inhibition Assay

Immunoglobulin G (IgG) was purified from sera of vaccinated mice using immobilized protein G (Thermo Scientific, Rockford, IL) and following the manufacturer's protocol. To evaluate the ability of RSV G polypeptide-specific antibodies to prevent RSV G protein binding to CX3CR1, 1 μg of purified serum IgG antibody was incubated with 1 μM of native G protein purified from either RSV A2 or B1 virus or with a control peptide, i.e. LH93 polypeptide (INGKWIILLSKF) for 1 h at 4°C. IgG purified from naive mouse serum was used as negative antibody control and MAb 131-2G was used as positive antibody control in all the assays. 293-CX3CR1 cells and untransfected 293 cells were plated in a round-bottom 96-well plate at 2 x 10^5 cells per well, washed with PBS and incubated with PBS containing anti-human CD32 (Fc block; Millipore) at 1 μg/ml, at 4°C for 15 min. After incubation, the cells were resuspended in pre-incubated mixture of purified IgG and native RSV G protein, and 5μg/ml of heparin (Sigma) was to prevent any nonspecific binding, and incubated for 1 hour at 4°C. After the incubation, the cells were washed in PBS containing 1% bovine serum albumin (fluorescence-activated cell

sorting [FACS] buffer) and incubated with MAb 130-2G conjugated to Alexa Fluor 488 (AF488) (Molecular Probes, Eugene, OR) for 30 min at 4°C. The percentage of G protein binding to 293-CX3CR1 or 293 cells only was determined by flow cytometry using a BD LSRII and FloJo analysis. The percent inhibition was calculated using the formula:

$$1 - \frac{(\%\text{ of 293-CX3CR1 AF488}^{+}\text{ cells treated with antibody mixture})}{(\%\text{ of 293-CX3CR1 AF488}^{+}\text{ cells treated with G protein or UV-inactivated RSV})}$$

RSV Plaque Reduction Assay

The ability of serum antibodies from RSV G polypeptide-vaccinated mice to neutralize RSV A2 or B1 virus was determined by plaque reduction assay. Briefly, 50 µl of 1ug/ml purified IgG from RSV G polypeptide-specific sera was mixed with RSV A2 or B1 virus that was previously titrated to yield 100 PFU/50 µl/well and was incubated for 2 hours at 37°C. Confluent monolayers of Vero E6 cells were prepared in 24-well plates (Corning, Corning, NY) and 100 ul/well of the IgG-RSV mixture added in triplicate. After virus adsorption for 2 h at 37°C, the cell monolayers were overlaid with 2.0% methylcellulose in MEM with 2% FBS at 100 ul/well. Plates were incubated at 37°C and 5% CO2 for 5 days. At day 5 post infection, the methylcellulose overlay was aspirated from the plate, the cells fixed with a 60% - 40% methanol-acetone mixture, and the fixed cells were air-dried overnight. The fixed cells were incubated with a mouse MAb specific for RSV F protein (clone 131-2A) followed by a secondary goat anti-mouse IgG antibody conjugated with alkaline phosphatase (KPL, Gaithersburg, MD). Plaques were developed using 200 µl/well of 1-STEP NBT/BCIP (Thermo Scientific, Rockford, IL) at room temperature for 10 min. Plaques were counted using a dissecting microscope. Titers were calculated from the averages of triplicate.

Statistics.

The data are presented as the mean ± the standard error of the mean (SEM). Student's *t* test was used to compare G polypeptide immunized groups and control groups. *P* values of < 0.01 were considered statically significant.

Results

Inhibition of Fractalkine binding to CX3CR1 in the presence of RSV native G protein

The effects of RSV G protein-CX3CR1 interaction on immunity and disease pathogenesis has been linked to G protein binding to the fractalkine receptor, CX3CR1, (291). Specifically, the presence of the RSV G protein CX3C motif has been shown to reduce the RSV-specific T cell responses via inhibiting CX3CL1-mediated trafficking of CX3CR1-expressing immune cells to the site of infection (123), and clinically, a variation in the CX3CR1 gene has been associated with increased risk for severe RSV bronchiolitis (266). Previous studies have only examined the effects of RSV G protein purified from RSV/A-infected Vero E6 cells (291). To determine if RSV/A and RSV/B G proteins mediate similar activities, and mimic the activities of fractalkine (FKN) for binding to CX3CR1, inhibition of FKN binding to CX3CR1 in the presence of native RSV/A2 or RSV/B1 native G proteins was examined (Table 1). The results show that RSV/A2 G protein inhibits FKN binding to CX3CR1 in a dose-response fashion, at a level similar to that previously shown (291), and that native RSV/B1 G protein showed similar dose-responsive inhibition of FKN binding to CX3CR1. These results suggest that RSV G protein from A and B strains have a similar capacity to mimic the activities of FKN.

Generation of RSV G polypeptide-specific polyclonal antibodies

Three RSV G polypeptides were used to immunize BALB/c mice to generate polyclonal antibodies corresponding to N-terminal variable, glycosylated region (G1 polypeptide, aa 67-147), the central conserved region (G2 polypeptide, aa 148-198), and the C-terminal hypervariable region (G3 polypeptide, aa 199-298) of the G protein of RSV strain A2 (Figure1). Mice were intramuscular immunized with 50 μg of G1, G2, or G3 polypeptide adjuvanted with TiterMax and equivalently boosted 2 weeks later. Three weeks following the second vaccination, sera from vaccinated mice were collected and pooled. Then IgG was purified and quantified from the pooled sera of vaccinated mice in order to normalize IgG levels and to remove any endogenous CX3CL1 or other serum factors that might affect downstream assays assessing G protein binding to CX3CR1. IgG antibodies purified from the sera of mice vaccinated with the G2 polypeptide (containing the central conserved region), or inactivated RSV/A2, reacted against both RSV A2 (Figure 2A) and RSV B1 (Figure 2B) native G proteins. In contrast, IgG antibodies purified from the sera of mice vaccinated with G1 or G3 polypeptide (comprising the N- and C-terminal variable regions, respectively) failed to recognize RSV A2 (Figure 2A) and RSV B1 (Figure 2B) native G proteins relative to negative control, suggesting that G2 polypeptide region that encompasses the CX3C motif is antigenic and can induce IgG antibodies that have some cross-reactivity with both RSV/A2 and RSV/B1 native G proteins.

RSV G polypeptide-specific antibodies inhibit G protein binding to CX3CR1.

Our laboratory previously reported that IgG antibodies purified from the sera of RSV/A2 G polypeptide immunized mice show various levels of blocking activity of RSV/A2 G protein binding to CX3CR1 (322). It has not been previously determined if antibodies to the G

polypeptide vaccines in this study (Figure 1) block native RSV/A as well as RSV/B1 G protein CX3C-CX3CR1 binding. To address this, G protein-CX3CR1 binding inhibition assays were performed with either native RSV/A2 (Figure 3A) or native RSV B1 (Figure 3B) G protein. Anti-RSV G protein monoclonal antibody (clone 131-2G) reactive to the central conserved region in the G protein (125, 128, 188), and IgG antibodies purified from naïve BALB/c mice were used as positive and negative controls, respectively. As expected, treatment of native RSV/A2 G protein with purified IgG from mice immunized with G2 polypeptides significantly ($p < 0.01$) inhibited G protein binding to CX3CR1 by approximately 40% (Figure 3A). A similar level of inhibition was observed following treatment of native RSV/A2 G protein with IgG antibodies purified from mice vaccinated with inactivated RSV/A2 virus, and predictably, treatment with monoclonal antibody 131-2G significantly ($p < 0.01$) inhibited G protein binding to CX3CR1 by approximately 85% (Figure 3A). Interestingly, IgG from mice immunized with G1 or G3 polypeptides also significantly ($p < 0.01$) inhibited G protein binding to CX3CR1, by approximately 30% (Figure 3A). These results suggest that antibodies generated to epitopes at or proximal to the G protein CX3C motif can reduce G protein binding to CX3CR1. Notably, treatment of RSV/B1 native G protein with purified IgG antibodies from G2 polypeptide vaccinated mice showed significant ($p < 0.01$) inhibition of G protein binding to CX3CR1; however, treatment with IgG antibodies purified from the mice vaccinated with G1 or G3 polypeptides had no substantial inhibitory effect (Figure 3B). In addition, IgG from inactivated RSV/A2 infected mice did not significantly inhibit RSV/B1 G protein binding to CX3CR1 (Figure 3B). These results indicate that the IgG antibodies generated to the central conserved region of the G protein can cross-react and inhibit G protein-CX3CR1 interaction.

RSV is neutralized by RSV G polypeptide-specific antibodies

RSV F and G proteins have been shown to induce neutralizing antibodies and confer protective immunity (53, 271), and studies that evaluated a BBG2Na vaccine candidate demonstrated a role for RSV G protein in protection against RSV disease (30, 63, 234). Moreover, a recent study that analyzed the serum from RSV A- and B-infected human subjects reported a substantial homo- and heterosubtypic IgG response to the central conserved region of the RSV G protein (199). To determine the level of antibody neutralization associated with RSV G polypeptide-specific IgG antibodies, purified IgG antibodies specific to G1, G2, or G3 polypeptides, or to inactivated RSV/A2, were tested for inhibition of RSV/A2 (Figure 4A) or RSV/B1 (Figure 4B) infection. While IgGs from G1 and G3 vaccinated mice did not exhibit any significant neutralizing antibody response against either strains of RSV, IgGs from G2 vaccinated mice showed significant ($P<0.01$) neutralizing ability against both RSV/A2 (60%) and RSV/B1 (35%) compared to the naïve IgG treatment. Meanwhile, IgGs from mice vaccinated with the inactivated RSV/A2 showed significant ($P<0.01$) neutralizing ability against RSV/A2 (80%) but did not show significant neutralization of RSV B1 compared to the native IgG treatment. Taken together, these results indicate that polyclonal IgG antibodies generated following vaccination of BALB/c mice with G2 polypeptide can neutralize RSV/A2, and provide a level of cross-neutralization against RSV/B1.

Discussion

Despite the significance of RSV as a leading agent of severe lower respiratory tract disease in infants and young children worldwide, there is still no safe and effective vaccination available. This study evaluates RSV G protein subunit vaccines composed of RSV G

polypeptides to induce antibodies that block G protein-CX3CR1 interaction. RSV is classified into two antigenic groups: A and B, with their differences located in the major attachment G protein (131, 132, 195). However, both RSV strains share the conserved central region containing CX3C chemokine motif which binds to fractalkine receptor, CX3CR1 (291). Interestingly, a previous study has demonstrated that a single nucleotide polymorphism in the CX3CR1 gene is associated with an increased risk for severe RSV bronchiolitis in children hospitalized for bronchiolitis (4). The G protein central conserved region containing the CX3C chemokine motif has been shown to have an important role in the modification of host immune responses during RSV infection, a feature that likely contributes to disease persistence and pathogenesis (123-126, 169, 289, 291). For example, a previous study from our group has shown that expression of the RSV G protein or the G protein CX3C motif during infection is associated with reduced $CX3CR1^{+}$ T cell trafficking to the lung, reduced frequencies of RSV-specific MHC class I-restricted IFNγ-expressing cells, and lower number of IL-4- and CX3CL1-expressing cells (123). Related to the study presented here, we have previously shown that BALB/c mice vaccinated with RSV G protein peptides containing the CX3C motif generated antibodies that inhibit RSV/A2 G protein-CX3CR1 interaction, reduce lung virus titers, and prevent body weight loss and pulmonary inflammation (322). These studies underscore the importance of G protein-CX3CR1 interaction in the modulation of host antiviral responses during RSV infection, and suggest a disease intervention targeted to inhibiting G protein-CX3CR1 interaction.

In the present study, to determine the ability of the RSV G protein-specific antibodies to inhibit RSV G protein-CX3CR1 interaction, BALB/c mice were vaccinated with three G polypeptides: G1 polypeptide (aa 67-147), G2 polypeptide (aa 148-198), and G3 polypeptide (aa 199-298) spanning the majority of the RSV G protein. We observed that, although all of the

vaccinated mice generated substantial humoral responses against the immunizing peptides, only the serum IgG purified from G2 polypeptide vaccinated mice strongly reacted the RSV A2 and RSV B1 native G proteins. The lack of antibody response against the RSV A2 and RSV B1 native G protein by G1 or G3 polypeptide vaccinated mice can may be explained by the highly glycosylated nature of the N-terminal and C-terminal regions (corresponding to the amino acid sequences represented by G1 and G3 polypeptides) within the RSV G protein that are flanking the central conserved region. Previous studies have found that the glycosylation pattern of the RSV G protein hypervariable regions changes with the infected cell type, indicating that the different glycosylation patterns on the RSV G proteins may be one of the mechanisms to evade the host immune response by altering the G protein antigenic profile (34, 97, 98, 221, 222). It is also possible that G1 and G3 polypeptides fold or aggregate differently compared to native G protein, and therefore differences may be associated with conformational epitopes that may not be present or are hidden in native G protein.

As we have previously shown, purified IgG antibodies from the sera of the RSV G polypeptide immunized mice have various levels of RSV G protein-CX3CR1 blocking activity for RSV A2 native G protein (322). However, in this study, we compared antibody efficacy for inhibiting both RSV A2 and B1 native G protein binding to CX3CR1 and inhibiting virus replication. Our results show that IgG antibodies purified from the sera of mice vaccinated with G2 polypeptide significantly inhibited RSV A2 and B1 native G protein binding to CX3CR1, and that IgG antibodies purified from the sera of G1- and G3- vaccinated mice also reduced RSV A2 G protein binding to CX3CR1. These results compare with a previous study from our laboratory which demonstrated that IgG antibodies from mouse vaccinated with G1, G2, and G3 polypeptides were able to significantly inhibit RSV A2 G protein-mediated leukocyte

chemotaxis, with G2-specific IgG showing the greatest inhibition (322). However, IgG antibodies purified from the sera of G1- and G3 vaccinated mice did not significantly inhibit RSV B1 G protein binding to CX3CR1. Previous antigenic relatedness studies of RSV G protein as determined by ELISA showed that the F proteins of RSV A and B strains have ~50% antigenic relatedness while the G proteins of two groups are more distantly related with ~5% antigenic relatedness (140). In the same study, the cotton rats immunized with the recombinant vaccinia virus expressing RSV/A2 G protein showed reduced heterosubtypic protection against RSV B strain compared to the cotton rats immunized with vaccinia virus expressing RSV/A2 F protein (140). Thus, the limited of inhibition of RSV B1 G protein binding to CX3CR1 by G1- and G3- polypeptide specific IgG antibodies may relate to known differences in the hypervariable regions of the G proteins. However, the lack of glycosylation in the central conserved region of the G protein shared by both RSV/A2 and RSV/B1 provides a target for G2-specific polypeptide IgG antibodies to bind and inhibit its interaction with CX3CR1.

In this study, it was evident that G2 polypeptide vaccination induced the highest level of antibodies reactive to both RSV A2 and B1 G proteins, while inactivated RSV vaccination also raised cross-reacting antibodies to RSV A2 and B1 G proteins (Figure 2). Despite anti-G2 polypeptide IgG antibody binding well to native A2 and B1 G protein, these antibodies did not cross-react effectively with RSV B1 G protein in the ELISA assay, and did not effectively inhibit RSV B1 G protein binding to CX3CR1 compared to antibodies to RSV/A2 G polypeptides. However, this is not unexpected because the ratio of anti- G, anti-F, and anti-SH protein-specific IgG antibodies raised against inactivated RSV is unknown. It is possible that the presence of anti-F and/or anti-SH antibodies may interfere with the binding of anti-G antibodies to their epitopes resulting in reduced inhibition of G protein-CX3CR1 interaction as was observed in the

CX3CR1 binding inhibition assay performed with RSV B1 G protein. Also, the anti-G antibodies raised to inactivated RSV A2 may react to other immunogenic epitopes on G protein but not specifically to the CX3C region thus showing reactivity against the RSV B1 G protein in ELISA while not effectively blocking the RSV B1 G protein-CX3CR1 interaction. Further, anti-G protein antibodies present in the sera raised against inactivated RSV A2 may be specific to other immunogenic epitopes (possibly conformational epitopes) within the G protein, but not specific to the CX3C motif, thus these antibodies may show reactivity against the RSV B1 G protein in ELISA while not effectively blocking the RSV B1 G protein-CX3CR1 interaction.

There is precedence that the RSV G protein can provide protection from challenge. A study evaluating a BBG2Na vaccine candidate in combination with different adjuvants and by different routes of administration has shown a role for the RSV G protein in protection against RSV in small and large animals (30, 63, 234). Furthermore, a study that analyzed the serum immunogenicity against various RSV G epitopes using sera from RSV A- and B-infected human subjects reported a significant increase in homo- and heterosubtypic IgG response against the central conserved region of the RSV G protein (199). Since, the neutralizing ability of antibodies is a good indication of protection against viral pathogens, the neutralizing ability of the RSV G polypeptide-specific IgG antibodies that block the RSV G protein-CX3CR1 interaction was determined using *in vitro* plaque reduction assay. The findings in this study showed that the polyclonal IgG antibodies generated from the vaccination of BALB/c mice with G2 polypeptide can effectively cross-neutralize both RSV/A2 and RSV/B1, indicating that the polyclonal IgG antibodies that are specific for the central conserved region of RSV A2 G protein may be able to offer heterosubtypic protection. These finds are consistent with an earlier study showing induction of cross-neutralizing antibodies induced by G protein expressed by

recombinant vaccinia which generated cross-neutralizing antibodies against both RSV/A and RSV/B strains (140). In addition, it has been shown that infants and children who are primarily infected with RSV A strain produced significant level of cross-neutralizing antibodies which could also neutralize RSV B strain (194). Also, when the response to the RSV G protein was examined in the same study, significant level of RSV B strain G protein-specific cross-neutralizing antibodies were detected in children infected with RSV A strain (194). The central conserved region of the RSV G protein is an immunogenic region that can generate RSV G protein-specific antibodies in humans which can inhibit RSV G protein-mediated leukocyte chemotaxis and interaction of the G protein CX3C motif with the chemokine receptor CX3CR1 (124). The results from this study shows that mice vaccinated with RSV G polypeptide containing the CX3C motif in the central conserved region generates IgG antibodies that block RSV A2 and RSV B1 G protein-CX3CR1 interaction and neutralize both A and B subtypes of RSV. The importance of these findings are that RSV G polypeptide vaccination can induce antibodies that recognize central conserved G protein peptide sequences shared by RSV A2 and B1 strains, and the reactivity of these antibodies can inhibit G protein binding to CX3CR1 and virus replication, an aspect which has not been previously described previously and is important in RSV vaccine development

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TABLE 1: Inhibition of fractalkine (FKN) binding to CX3CR1 by RSV native G protein. 293-CX3CR1 cells were incubated with 1 μg, 500 ng, or 250 ng of RSV A2 or RSV B1 native G protein in the presence of 5 μg/ml heparin. Cells were then treated with 1 μg of 5FAM-conjugated fractalkine peptide, and the FKN peptide binding to 293-CX3CR1 cells was determine by FACS analysis. The percent inhibition of FKN peptide binding to 293-CX3CR1 cells by RSV A2 or RSV B1 native G proteins was calculated and shown relative to the percent FKN peptide binding on 293-CX3CR1 cells in the absence of RSV native G protein.

Inhibitors	% FKN Inhibition
RSG A2 G protein (1ug)	54.5% - 46.7%
RSV A2 G protein (500ng)	51.0% - 39.8%
RSV A2 G protein (250ng)	45.3% - 34.6%
RSV B1 G protein (1ug)	57.9% - 50.4%
RSV B1 G protein (500ng)	47.8% - 37.3%
RSV B1 G protein (250ng)	30.0% - 21.3%

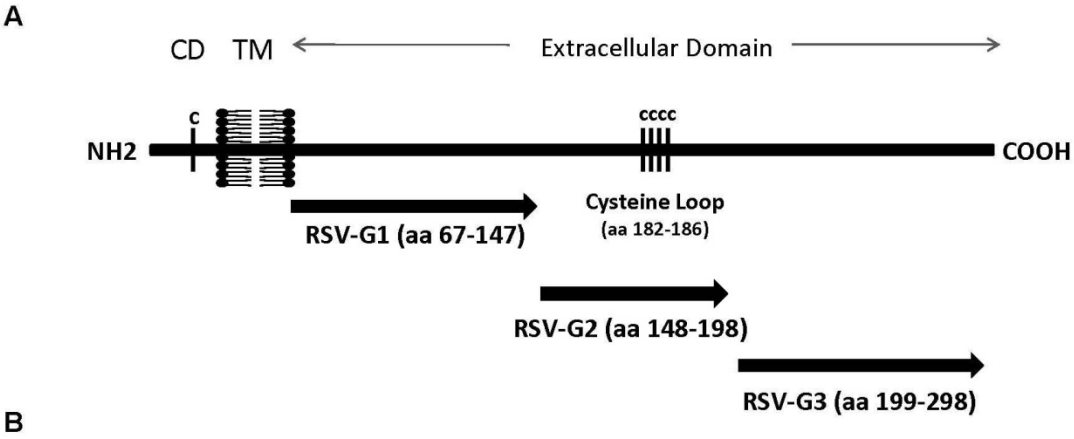
A
CD
TM
Extracellular Domain
C
CCCC
NH2
COOH
Cysteine Loop
(aa 182-186)
RSV-G1 (aa 67-147)
RSV-G2 (aa 148-198)
RSV-G3 (aa 199-298)
B
RSV-G2 PPT
148
169
182
186
198
TKQRQNKPPNKPNNDFHFEVFNFVPCSICSNNPT C W A I C KRIPNKKPGKKT
RSV A2
NFVPCSICGNNQL C K S I C KTIPSNKPKKKP
RSV B1
169
182
186
198

FIGURE 3.1. Schematic of RSV G protein and location of G polypeptides. (A) The transmembrane region and cytoplasmic domain are indicated by TM and CD, respectively. The cysteine loop region is indicated. The locations of the RSV G polypeptides on the G protein, i.e. G1, G2, and G3 are also depicted. (B) Amino acid sequence of G2 polypeptide (PPT) is shown. For the purpose of comparison, the amino acid sequence of the cysteine loop region of the RSV B1 G protein (aa 169-198) is also depicted. The CX3C motifs in RSV A2 and B1 G proteins are underlined and the non-homologous amino acid residues are highlighted in bold.

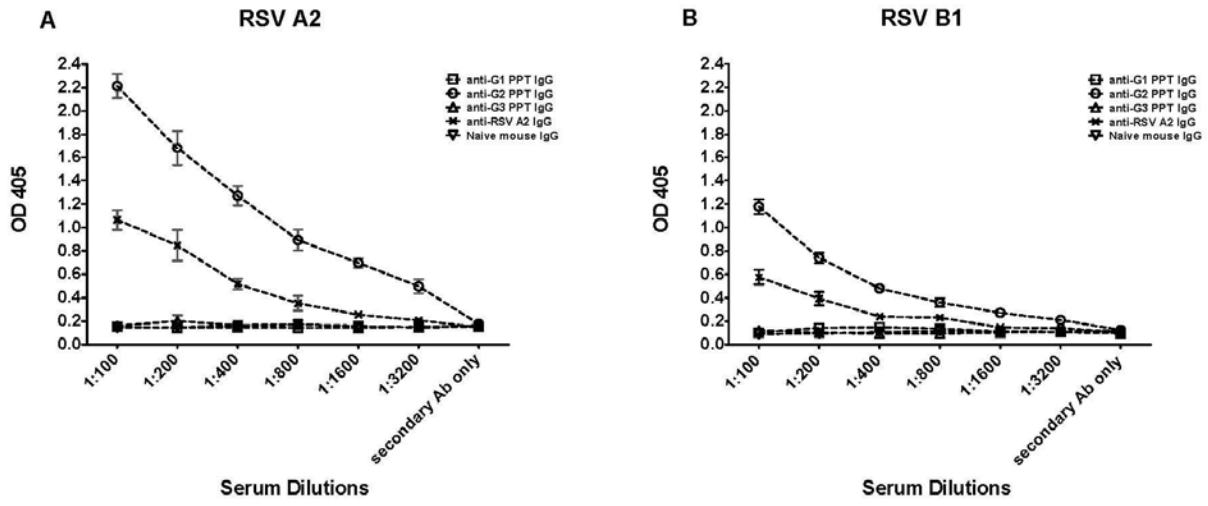
A
RSV A2
OD 405
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
anti-G1 PPT IgG
anti-G2 PPT IgG
anti-G3 PPT IgG
anti-RSV A2 IgG
Naive mouse IgG
1:100
1:200
1:400
1:800
1:1600
1:3200
secondary Ab only
Serum Dilutions
B
RSV B1
OD 405
anti-G1 PPT IgG
anti-G2 PPT IgG
anti-G3 PPT IgG
anti-RSV A2 IgG
Naive mouse IgG
1:100
1:200
1:400
1:800
1:1600
1:3200
secondary Ab only
Serum Dilutions

FIGURE 3.2. RSV G polypeptide-specific IgG antibodies are reactive against native RSV/A2 and RSV/B1 G proteins. Purified IgG antibodies from the serum of G polypeptide (PPT) G1, G2, G3, or UV-inactivated RSV/A2 mice were evaluated for reactivity to native RSV/A2 (A) or native RSV/B1 (B) G proteins. The results represent three independent assays with three replicates per dilution per assay +/- SEM.

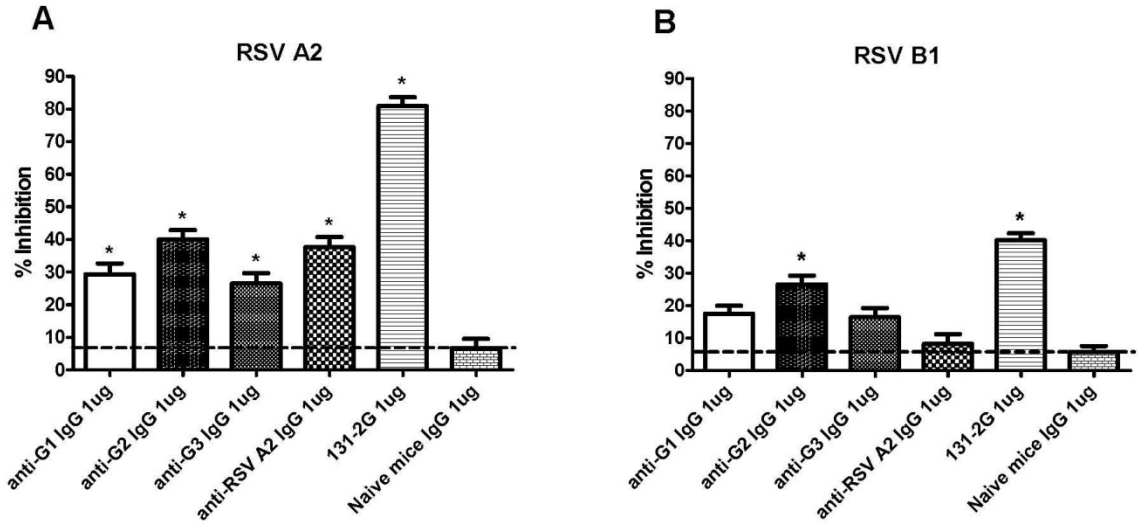
A
RSV A2
% Inhibition
90
80
70
60
50
40
30
20
10
0
anti-G1 IgG 1ug
anti-G2 IgG 1ug
anti-G3 IgG 1ug
anti-RSV A2 IgG 1ug
131-2G 1ug
Naive mice IgG 1ug
B
RSV B1
% Inhibition
anti-G1 IgG 1ug
anti-G2 IgG 1ug
anti-G3 IgG 1ug
anti-RSV A2 IgG 1ug
131-2G 1ug
Naive mice IgG 1ug

FIGURE 3.3. Purified IgG antibodies from G2 polypeptide vaccinated mice inhibit RSV/A2 and RSV/B1 native G protein binding to CX3CR1. IgG antibodies purified from the sera of G polypeptide- or UV-inactivated RSV A2-vaccinated mice were examined for their ability to inhibit binding of purified RSV/A2 or RSV/B1 native G protein to 293-CX3CR1 cells. Data represents the percent inhibition of RSV/A2 native G protein (A) or RSV/B1 native G protein (B) binding to 293-CX3CR1 cells. RSV G protein-specific monoclonal antibody (clone 131-2G) and IgG purified from naïve mouse sera are positive and negative controls, respectively. The percent inhibition was calculated using the formula: [1 – (percent Alexa488 positive of 293-CX3CR1 treated with the antibody mixture / percent Alexa488 positive of 293-CX3CR1 treated with G protein only)]. * indicates $P<0.01$ (when compared with naïve mouse IgG). The results represent three independent assays with three replicates per dilution per assay +/- SEM.

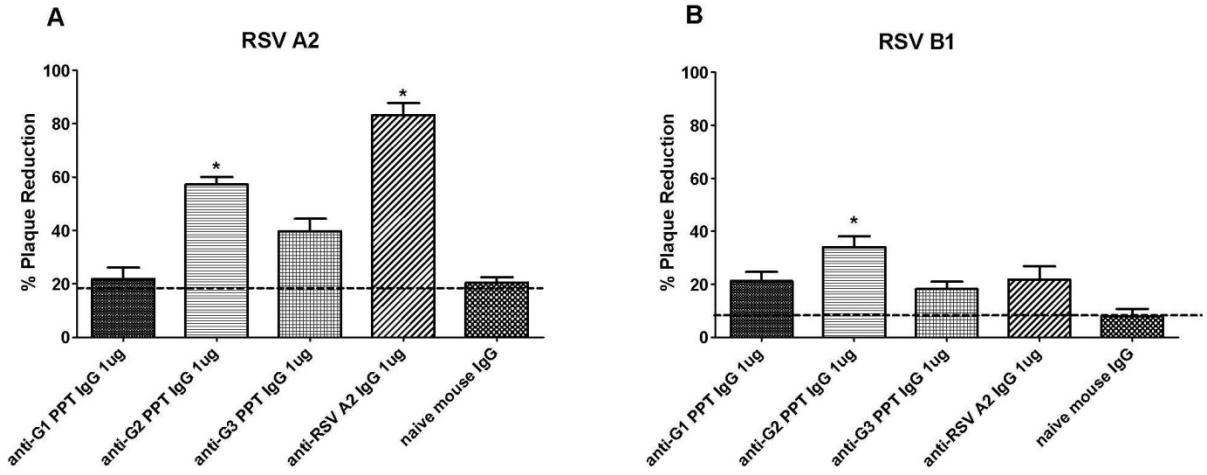
A
RSV A2
% Plaque Reduction
100
80
60
40
20
0
*
*
anti-G1 PPT IgG 1ug
anti-G2 PPT IgG 1ug
anti-G3 PPT IgG 1ug
anti-RSV A2 IgG 1ug
naive mouse IgG
B
RSV B1
% Plaque Reduction
100
80
60
40
20
0
*
anti-G1 PPT IgG 1ug
anti-G2 PPT IgG 1ug
anti-G3 PPT IgG 1ug
anti-RSV A2 IgG 1ug
naive mouse IgG

FIGURE 3.4. Neutralization of RSV A2 and RSV B1 by IgG antibodies purified from sera of RSV G polypeptide-vaccinated mice. Purified RSV G polypeptide (G1, G2, G3)-specific IgG antibodies were examined for their ability to neutralize RSV A2 (A) and RSV B1 (B) by plaque reduction assay. Vero E6 cells were infected with 10^2 PFU of live RSV A2 or B1 stain that was previously incubated with 1ug per well concentration of various RSV G protein-specific IgG antibodies. At day 5 post-infection, cells were fixed with acetone:methanol (60:40). The plaques were immunostained using RSV anti-F monoclonal antibody 131-2A and counted. RSV F protein-specific monoclonal antibody from clone 131-2A and PBS were used as positive and negative controls, respectively. Data are represented as the percent RSV plaque reduction and the values are expressed as percent plaque reduction relative to the negative control. * indicates $P<0.01$ (when compared with naïve mouse IgG).

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CHAPTER 4

NANOPARTICLE SUBUNIT VACCINES EXPRESSING THE RSV G PROTEIN CX3C CHEMOKINE MOTIF INDUCE ROBUST B AND T CELL IMMUNE RESPONSES PROTECTING FROM RSV CHALLENGE

Youngjoo Choi, Patricia A. Jorquera, Thomas J. Powell, Lia M. Haynes, Larry J. Anderson, and Ralph A. Tripp. To be submitted to Vaccine

Abstract

Nanoparticle vaccines expressing the CX3C motif from the G glycoprotein of the respiratory syncytial virus were synthesized via layer-by-layer (LbL) assembly technique. BALB/c mice immunized with the G protein nanoparticle vaccines were immunogenic and produced neutralizing antibody response and exhibited reduced virus replication in the lungs following RSV A2 challenge. Additionally, ELISPOT analysis of spleens demonstrated that, following RSV A2 challenge, nanoparticle vaccinated mice significantly increased the level of RSV G-specific IL-4 and IFN-γ secreting cells compared to the unvaccinated control. Nanoparticle vaccination was also associated with the increased level of RSV M2-specific IL-4 and IFN-γ in the spleen. Further, our results from RSV M2-specific H-2Kd-tetramer staining revealed that nanoparticle vaccinated mice exhibited significant increase in the recruitment of RSV M2-specific CD8+ T cells to the lungs compared to the unvaccinated control. Cell type analysis by showed no significant increase in neutrophil ($Ly6G^{high}$ $Ly6C^{int}$ $CD125^{low}SiglecF^{-}$) or eosinophils ($Ly6G^{int}$ $Ly6C^{high}$ $CD125^{high}$ $SiglecF^{+}$) population in the lungs of the vaccinated mice following RSV A2 challenge. Taken together, our results demonstrate that vaccination of mice with RSV G nanoparticle vaccines induce neutralizing antibody response, increase RSV G- and M2- specific T lymphocyte responses, and may reduce RSV granulocyte-mediated disease pathogenesis.

Introduction

Human respiratory syncytial virus (RSV) is an ubiquitous negative sense, single-stranded RNA (ssRNA) virus in the Paramyxovirus family that causes serious lower respiratory tract disease in infants, elderly, and the immune compromised (17, 22, 31, 47). Natural infection with RSV provides limited protection from reinfection as an individual can experience recurrent RSV infection throughout life (16, 30). Although substantial efforts have been made toward developing a safe and effective RSV vaccine, none have been successful due to the difficulties in achieving correct balance of safety and efficacy. The first RSV candidate vaccine was the formalin-inactivated RSV (FI-RSV), which did not confer protection and and led to an enhanced disease characterized by pulmonary eosinophilia upon subsequent natural RSV infection (11, 91). Various live-attenuated RSV vaccine candidates have also failed confer protection due to a difficulty in achieving an appropriate level of attenuation as direct correlation seems to exist between the replicative ability of the vaccine virus and the immunogenicity (46, 48, 50, 61, 92). Given these findings, the development of subunit vaccines against RSV appears more promising.

Several RSV subunit vaccine formulations have been examined including purified F protein (PFP) vaccines (4, 7, 23, 29, 37, 44) and those targeting G protein. Evidences provided by previous studies indicate that the RSV F protein is important in inducing protective immunity (29, 49). Accordingly, subunit vaccines based on the RSV F protein isolated from infected cell cultures have been extensively evaluated in humans of various age groups (7, 23, 29, 44, 57, 82). Although the F protein-based vaccine candidates were well tolerated by the human subjects, they were not sufficiently immunogenic (7, 23, 29, 44, 57, 82). Meanwhile, studies have shown that a G protein subunit vaccine candidate, BBG2Na, elicits robust immune responses in small and large animals (8, 15, 63) and has been evaluated in human clinical trials. BBG2Na is a fusion

protein, synthesized in bacteria, consisting of the central conserved region of the G protein from RSV A Long strain (amino acid sequence 130-230) annealed to the C-terminus of an albumin-binding region of the streptococcal G protein (8, 15, 63). The phase I trials, performed with 108 healthy young adults proved that BBG2Na is both immunogenic and protective (62). However, the vaccine studies discontinued shortly after due to unexpected development of type III hypersensitivity (purpura) in two healthy young adults during phase II trials (19, 59). Moreover, a recent study using virus-like particles (VLPs) demonstrated that mice immunized with VLPs carrying RSV F or G protein produced higher viral neutralizing antibody levels and exhibited significantly decreased lung virus titer following live RSV A2 challenge. Interestingly, this study demonstrated that RSV-G VLPs vaccination conferred better protection against RSV challenge compared to RSV-F VLPs vaccination as determined by the reduction in lung virus titers and morbidity post-challenge (65).

Despite the evidences indicating that RSV G protein plays an important role in the induction of protective immunity, G protein has also been implicated in RSV disease pathogenesis (36, 51, 54, 75). Previous studies have described that RSV G protein possesses immuno-modulatory functions that are capable of modifying the innate and adaptive immune responses of the infected host (33, 77, 79, 81). One of the mechanisms that G protein utilizes to dysregulate host immune responses is the CX3C chemokine mimicry (78). The CX3C chemokine mimicry is attributed to the central conserved region of the G protein which contains four cysteine residues (1, 33, 40, 58, 78), as this region contains marked similarity to the receptor binding region of fractalkine, the only known CX3C chemokine, and possesses fractalkine-like leukocyte chemotactic activity (78). As such, RSV G protein functions as a fractalkine antagonist, modulating the immune response to infection by inhibiting fractalkine-mediated

trafficking of CX3CR1-expressing immune cells and modifying the magnitude and cadence of cytokine and chemokine expression (33, 76). Interestingly, a previous study by our group has shown that vaccination with RSV G polypeptides spanning the central conserved region of the G protein induce antibodies that blocked G protein-CX3CR1 interaction and reduce disease pathogenesis mediated by RSV infection, suggesting that RSV G polypeptide vaccination approach to generate antibodies specific to the central conserved region of the G protein that block RSV G protein-CX3CR1 interaction may be a sound strategy in developing safe and effective RSV vaccine that prevent RSV G protein-mediated immune evasion and disease pathogenesis (94).

Utilizing synthetic nanoparticles as a mechanism to deliver antigenic peptides for immunization is an attractive strategy to develop novel vaccines against various infectious diseases. Nanoparticle vaccines can be designed to closely mimic the structures of viral and bacterial pathogens. Immune cells can thereby recognized this mimicry of particle structures and induce robust immune responses against the nanoparticles even in the absence of conventional adjuvant. One way of creating nanoparticles for vaccination is layer-by-layer (LbL) assembly of polypeptides. This technique involves a sequential overlaying of oppositely charged polypeptides onto an internal core, such that the oppositely charged polypeptides are deposited on the top of each other via electrostatic interactions. Using LbL assembly technique, nanoparticles with various functions and characteristics can be engineered due to the flexibility in size, shape, and composition of the internal core substrate as well as the choice of polypeptides that can be used to layer the nanoparticle. For example, LbL assembly technique can be used to produce artificial red blood cells (56), controlled-release particles loaded with insulin and vitamins (13, 24). Meanwhile, this technique can also be utilized to produce virus-

like particles expressing immunogenic epitopes to be used as a platform for vaccine delivery (55). Previous studies that have investigated the possible utilization of LbL nanoparticles as the vaccine delivery mechanism reported that vaccination with the layer-by-layer nanoparicles incorporated with well-defined antigenic epitopes improved both humoral and cell-mediated immune responses in OVA (25, 26, 83, 84), hepatitis B (41), tumor (32, 69), and RSV (43) models. Such robust immunogenicity generated by the nanoparticle vaccination is attributed to more efficient stimulation of innate immunity (39, 85, 93). Previous studies have observed increased efficiency in phagocytosis, cross-presentation, and activation of dendritic cells (DCs) measured by cytokine production and costimulatory marker expression following LbL nanoparticle vaccination (39, 85, 93). With its flexibility in design which allows the manipulation of particle size and the contents of the incorporated antigenic peptides to engineer the construct that generate the desired immune responses, LbL nanoparticle technology is a promising candidate for the production of delivery mechanism for future RSV subunit vaccines.

In the present study, we adapted LbL nanopariticle technology to produce synthetic nanoparticles carrying one of the three designed target polypeptides comprising of the CX3C motif from the RSV G protein of the RSV A2, B1 and NY/CH17/83 strains (GA2, GB1 and GCH17). Our results show that vaccination of BALB/c mice with the RSV G nanoparticle vaccines elicits neutralizing antibody response, and is associated with increased in RSV G- and M2- specific CD4+ and CD8+ T lymphocyte responses and reduction in RSV disease pathogenesis following RSV A2 challenge. Taken together, our findings demonstrate that the vaccination approach using LbL nanoparticles engineered to carry the RSV G protein CX3C motif may be a promising strategy in developing a novel RSV vaccine that is effective in preventing RSV G protein-mediated immune modulation and disease pathogenesis.

Materials and Methods

Animals

Specific-pathogen-free, 6-to-8 weeks old female BALB/cAnN (H-2^d) mice (National Cancer Institute, NCI) were used in all experiments. Mice were housed in microisolator cages and were fed sterilized water and food *ad libitum*. All experiments were performed in accordance with the guidelines of the University of Georgia Institutional Animal Care and Use Committee.

Virus and infection

The A2 and B1 strains of RSV were propagated in Vero E6 cells (ATCC CRL-1586) as described elsewhere (79). Mice were anesthetized by intraperitoneal administration of Avertin (2,2,2-tribromoethanol 0.2 mL/g body weight; Sigma-Aldrich) and intranasally challenged with 10^6 PFU of A2 strain of RSV in serum-free Dulbecco modified Eagle medium (DMEM)/ high glucose (*HyClone*, ThermoFisher Scientific).

Peptide synthesis

Peptides spanning the G protein CX3C motif of the RSV strains A2, NY/CH17/83 and B1 were designed for vaccination (Table 4.1). C-terminal amide peptides were synthesized on a CEM Liberty^{TM} microwave assisted synthesizer using the manufacturer's standard synthesis protocols. Crude reduced peptides were partially purified by C_{18} reversed phase HPLC, correct molecular weight was confirmed by electrospray mass spectrometry (ESMS), and then lyophilized. Oxidative refolding was accomplished by dissolving the peptides at 2-5 mg/mL in redox buffer (2.5 mM reduced glutathione, 2.5 mM oxidized glutathione, 100 mM Tris pH 7.0) for 3 h at room temperature then at 4°C overnight. Folding was judged complete by a shift to

slightly shorter retention time on analytical HPLC. Following a final HPLC purification step refolding was confirmed by a loss of 4.0 (+/- 0.4) amu in the ESMS spectra relative to that of the reduced peptide, as well as an absence of free thiol as detected by DTNB (Ellman's) assay. Correct disulfide bonding was partially confirmed by ESMS of fragments generated from a thermolysin digest (27) of the synthetic peptide (data not shown). Peptides were aliquoted, lyophilized, and stored at -20°C until use.

Nanoparticle fabrication and quality control

Nanoparticles were constructed as previously described (60) on 50 nm diameter $CaCO_3$ cores by alternately layering poly-l-glutamic acid (PGA, negative charge) and poly-l-lysine (PLL, positive charge) to build up a seven-layer film; designed peptide (DP) containing the RSV G protein CX3C motif linked to a cationic sequence was added as the outermost layer. The compositions of the films were determined by amino acid analysis (AAA) which showed that comparable amounts of the three peptide components were present in each batch. Endotoxin levels were measured using limulus amebocyte lysate (LAL) assay and were found to be less than 0.1 EU/ug of G peptide. The dispersity of the particle vaccines was monitored by dynamic light scattering (DLS). Stepwise LbL steadily increases the diameter of the particles several fold, from an apparent diameter of about 150 nm for uncoated particles to about 400-500 nm for fully coated particles. Some particle aggregation was detected in each batch with a second population of particles in the 1500-2000 nm range.

Vaccination

LbL nanoparticles were suspended in sterile phosphate buffered saline (PBS; HyClone, ThermoFisher Scientific) and dispersed by water bath sonication immediately prior to immunization. Doses were adjusted to deliver either 10 μg or 50 μg DP/100 μL/mouse. Mice were immunized subcutaneously (s.c.) between the shoulder blades on day 0 and boosted on day 21 with the same dose of vaccine used during prime immunization. Control mice received 100 μL of PBS per injection. The antisera from control and nanoparticle-vaccinated mice were collected at 21 days post boost immunization and stored at -80°C until use.

Indirect ELISA.

RSV/A2- and B1-specific IgG antibodies were detected by ELISA on 96-well high binding plates (Corning, NY) coated with 10^6 PFU/mL RSV/A2 or /B1 in 0.05 M carbonate-bicarbonate buffer, pH 9.6 overnight at 4°C. Sera were added to plates in serial dilutions. RSV-specific antibodies were detected with horseradish peroxidase (HRP) conjugated antibodies specific for mouse IgG, mouse IgG1 or mouse IgG2a (SouthernBiotech) followed by addition of SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL, Inc.) for 15 min. Antibody titers were determined as the last sample dilution that generated an OD_{450} reading of greater than 0.2.

RSV plaque inhibition assay

Sera obtained from vaccinated and naïve mice were heat inactivated at 56°C for 30 min, and serial two-fold dilutions starting at a dilution of 1:20 were made in DMEM containing 2% heat-inactivated fetal bovine serum (FBS). Equal volumes of serum dilutions and RSV strain A2

previously titrated to yield 200 PFU/100 µL/well of final mixture were incubated at 37°C and 5% CO_2 for 1 h. Confluent monolayers of Vero E6 cells prepared in 24-well plates were infected with 200 µL/well, in triplicate, of the serum-virus mixture. After virus adsorption for 2 h at 37°C, the cell monolayers were overlaid with 2% methylcellulose media (DMEM, supplemented with 2% fetal bovine serum and 2% methylcellulose). Plates were incubated at 37°C and 5% CO2 for 5 days. The cells were then fixed with ice-cold acetone:methanol (60:40) and incubated with a mouse monoclonal antibody specific for RSV F protein (clone 131-2A) followed by a secondary goat anti-mouse IgG antibody conjugated to alkaline phosphatase (AP) (Invitrogen). Plaques were developed using 200 µL/well of 1-Step™ NBT/BCIP (ThermoFisher Scientific) at room temperature for 5 to 10 min. Plaques were counted using a dissecting microscope. Titers were calculated from the averages of triplicate sample wells and expressed as the percent plaque inhibition relative to that of the naïve serum control.

Lung virus titers

RSV lung virus titers in vaccinated and control mice were determined as previously described (79). Briefly, lungs were aseptically removed from anesthetized mice at day 5 post-RSV/A2 challenge (10^6 PFU /mouse), and individual lung specimens were homogenized at 4°C in 1 mL of serum-free DMEM/ high glucose (HyClone, ThermoFisher Scientific) by use of gentleMACS™ Dissociator (Miltenyi Biotec). Samples were centrifuged for 10 min at 200 x g, the supernatants were transferred to a new tube and used immediately or stored at -80°C until they were assayed. For the plaque assay, 10-fold serial dilutions of the lung homogenates were added to 90% confluent Vero E6 cell monolayers. Following adsorption for 2 hours at 37°C, cell monolayers were overlaid with 2% methylcellulose media and incubated at 37°C for 5 days. The

plaques were enumerated by immunostaining with monoclonal antibodies against RSV F protein (clone 131-2A) as described above.

IFN-γ and IL-4 ELISPOT analysis

Multiscreen 96-well plates (Millipore) were coated with the anti- mouse IL-4 or anti-mouse IFNγ capture antibody (R&D Systems) and incubated overnight at 4°C. The plates were then blocked by the addition of 200 µL of RPMI-10 media (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol and 2 mM L-glutamine) and incubated for 2 h at 37°C. In parallel, spleens were harvested from vaccinated and naïve mice at 5 days post RSV/A2 challenge and prepared to a single cell suspension using a syringe plunger and a 70 µm mesh nylon strainer. The cell suspensions were collected by centrifugation for 10 min at 200 x g and suspended in RPMI-10 at a concentration of 10^7 cells/mL. A total of $5x10^5$ cells were added to each well, and cells were stimulated with either 5 µg/mL $M2_{82-90}$ (SYIGSINNI), 5 µg/mL $G_{183-197}$ (WAICKRIPNKKPGKK) or without peptide for 24 h at 37°C and 5% CO_2. Plates were washed 4 times with wash buffer (0.05% Tween-20 in PBS), anti-mouse IL-4 or anti-mouse IFNγ biotin-conjugated detection antibody (R&D Systems) was added and plates were incubated overnight at 4° C. After incubation, plates were washed, cytokine spots were developed using ELISpot blue color module (R&D Systems) and counted using an ELISPOT reader (AID EliSpot Reader System). RSV-specific ELISPOT numbers were determined from triplicate wells/cell population by subtracting the mean number of ELISPOTs in the unstimulated wells. For statistical evaluation, t test for unpaired samples was used to compare the number of RSV-specific ELISPOTs with the number of ELISPOTs induced by RMPI-10 media. Values of $p < 0.05$ were considered significant.

Flow cytometry for lung cells phenotype analysis

Lungs were aseptically removed from mice at day 5 post- RSV challenge, and individual lung specimens were homogenized at 4°C in 1 mL of serum-free DMEM/ high glucose (HyClone, ThermoFisher Scientific) by use of gentleMACS™ Dissociator (Miltenyi Biotec). Samples were resuspended in RPMI-10 media and filtered through a 70 µm mesh nylon strainer. Cell suspensions were centrifuged for 10 min at 200 x g and then washed with staining buffer (PBS containing 1% BSA). For MHC tetramer staining, cells were stained with PE-Cy7-conjugated hamster anti-mouse CD3e (145-2C11), PerCP-Cy5.5-conjugated rat anti-mouse CD8α mAb (53-6.7), and optimized amount of APC-conjugated MHC class I H-2K^{d} tetramer complexes bearing the peptide SYIGSINNI representing the immunodominant epitope of the RSV M2-1 protein (38). To determine cell types in lungs, cell suspensions were stained for 30 minutes at 4° C with the optimized amount of PE-Cy7-conjugated anti-Ly6C (AL-21), PerCP-Cy5.5-conjugated anti-Ly6G (1A8), PE-conjugated anti- SiglecF (E50-2440), Alexa fluor 488-conjugated anti-CD125 (T21), and purified anti-CD16/CD32 (all mAbs were obtained from BD Biosciences). After staining, cells were washed and resuspended in staining buffer. Analysis of cell surface marker expression was performed using a BD LSRII flow cytometer (BD bioscience). A total of 100,000 events were analyzed per sample. Based on cell surface markers expression three different cell type were identified: Ly6G$^{intermediate\ (int)}$ Ly6C^{high} CD125^{high} SiglecF^{+} as eosinophils, Ly6G^{high} Ly6C^{int} CD125^{low}SiglecF^{-} as neutrophils and Ly6G^{int} Ly6C^{int} CD125^{low}SiglecF^{+} as macrophages (14, 66, 70, 71, 74, 95).

Statistics

All statistical analyses were performed using GraphPad software (San Diego, CA). Statistical significance was determined using an unpaired Student's t test. A p value < 0.05 was considered statistically significant.

Results

RSV G nanoparticle vaccination induces robust antibody responses

In order to generate a nanoparticle vaccine carrying the CX3C motif of the RSV G protein, $CaCO_3$ nanoparticle cores were layered with poly-l-glutamic acid (PGA) and poly-l-lysine (PLL) consisting of eight total layers with the designed peptide (DP) inserted at the eighth layer. Three different nanoparticles were generated: GA2, GCH17 and GB1 (Table 4.1). Groups ($n = 5$) of BALB/c mice were immunized via subcutaneous injection with 10 μg or 50 μg dose of GA2, GCH17 or GB1 nanoparticle in PBS. Age- and sex-matched BALB/c mice that were immunized with PBS were used as negative control. Animals received a boost of the same volume and concentration at days 21 post-prime. At day 21 post-boost, mice were bled for determination of antibody titers by ELISA using plates coated with either RSV/A2 or RSV/B1. Immunization with either dose of GA2 or GCH17 nanoparticle vaccine elicited high titers of anti-RSV/A2 IgG (Fig.4.1A) and lower but significant levels of cross-reactive anti-RSV/B1 IgG (Fig.4.1B). Interestingly, RSV A2-specific IgG production reached maximum levels at 10 μg dose for GA2 and GCH17 vaccines, but 50 μg dose was required to induce an adequate level of cross-reactive IgGs for B1 vaccine. Nanoparticle vaccination elicited both IgG1 and IgG2a isotypes, with IgG1 titers being higher than IgG2a titers (Fig.4.1C – 4.1F). However, higher levels of IgG2a were induced in groups that received 50 μg dose. These results demonstrate that

vaccination with RSV G nanoparticles in the absence of adjuvant can elicit a strong humoral response comprising both IgG1 and IgG2a isotypes.

Vaccination with RSV G nanoparticle vaccines induces neutralizing antibodies

Neutralizing antibody is an important functional component of immune responses induced by vaccination. In order to determine whether vaccination with RSV G protein nanoparticle vaccines induce neutralizing antibodies, an *in vitro* RSV plaque reduction assay was performed using heat-inactivated mouse sera from vaccinated or challenged mice (Fig.4.2). At twenty-one day post-boost but prior to challenge, all vaccinated mice showed modest levels of neutralizing antibodies against RSV/A2 (20-35% plaque reduction), with GB1 vaccinated mice having the weakest response (<12% plaque reduction) (Fig.4.2A). However, following RSV A2 challenge, two- to four-fold increase in the neutralizing ability was observed by the sera collected from GA2 ($p < 0.001$) and CH17 ($p = 0.004$) vaccination groups (Fig. 4.2B). Also, a slight increase in the neutralizing ability was observed in GB1 ($p = 0.0123$) vaccination group as well (Fig.4.2B). These results indicate that vaccination with RSV G nanoparticles induces a robust humoral response which leads to high levels of RSV-specific and cross-neutralizing antibodies and an effective B cell memory response that can be promptly activated upon RSV infection.

RSV G nanoparticle vaccination is protective against RSV challenge

In order to evaluate whether RSV G nanoparticle vaccination can induce protective immunity against RSV infection, vaccinated mice were challenged intranasally with RSV/A2. Each mouse received 1 x 10^6 PFU of challenge virus at week 6 post-boost, and the lung virus

titer was determined at day 5 post-challenge. Animals vaccinated with GA2, GCH17 and GB1 nanoparticle vaccines showed significant ($p < 0.05$) decrease in the lung virus titers compared to the group that was immunized with PBS (Fig.4.3). Importantly, mice vaccinated with GB1 nanoparitcles also demonstrated significant ($p < 0.05$) reduction in the lung virus titer, suggesting that GB1 vaccination induced cross protection against RSV/A2 challenge. Also, a dose-dependent response was detected in animals vaccinated with GA2 or GB1 nanoparticles as the 50 μg dose was associated with lower lung virus titers compared to the 10 μg dose. However, no dose-dependent reduction in the lung virus titer was observed in mice vaccinated with GCH17 nanoparticles. Overall, these results suggest that vaccination of mice with RSV G nanoparticles can induce a significant inhibition of RSV replication in the lungs of infected host.

Mice vaccinated with RSV G nanoparticles develop non-biased Th1/Th2 responses

Previous studies have reported that priming BALB/c mice with RSV G protein can elicit a Th2-biased CD4+ T lymphocyte responses upon subsequent challenge with RSV (52, 53, 75, 76), and the resulting predominant Th2 type responses have been suggested to mediated the manifestation of disease symptoms such as airway hyperresponsiveness, mucus over-production, and pulmonary eosinophilia during RSV infection (28, 86-89). In order to address this concern, we investigated whether RSV G nanoparticle vaccination induces Th2-biased T lymphocyte responses in the immunized mice. Accordingly, RSV G nanoparticle vaccinated mice were challenged intranasally with RSV/A2 at week 6 post-boost, and the Th1- (IFNγ) and Th2- (IL-4) type lymphocyte frequencies were determined by ELISPOT assay using the spleens of challenged animals harvested at day 5 post-challenge. Compared to the PBS vaccinated control, GA2, GCH17 or GB1 nanoparticle vaccinate mice responded to live RSV challenge by inducing

a significantly augmented T lymphocyte responses against RSV G peptide epitopes ($G_{183\text{-}197}$; WAICKRIPNKKPGKK) consisting of both Th1- (IFNγ+) and Th2- (IL-4+) type populations (Fig.4.4 A and B). Interestingly, animals that were vaccinated with 50 μg of GA2, GCH17 and GB1 nanoparticles were associated with a significant ($p < 0.05$) increase in the M2-specific IFNγ-secreting splenocytes compared to that of the PBS vaccinated control (Fig.4.4D). Further, a mild increase in the M2-specific IL-4-secreting splenocytes frequency was generally observed in the RSV G nanoparticle vaccinated animals (Fig.4.4C). Since the nanoparticle vaccines were not carrying any RSV M2 peptide sequences, we hypothesize that the observed M2-specific responses were elicited by the RSV challenge. Taken together, these results indicate that RSV G nanoparticle vaccination not only induces a potent, Th1/Th2 non-biased, G-specific lymphocyte responses but also potentiates the RSV M2-specific primary T lymphocyte responses following live virus challenge.

Vaccination with RSV G nanoparticles potentiates M2-specific CD8 T cell responses

To evaluate if the increase in frequency of M2-specific IFNγ-secreting cells was due to an incremental change of the population of M2-specific $CD8^+$ T cells, total spleen and pulmonary cells from mice vaccinated with 50 μg of G nanoparticles were isolated on day 5 post-challenge and analyzed to quantify the number of $CD8^+$ T cells that were positive for binding to the RSV $M2_{82\text{-}90}$ MHC class I H-$2K^d$ tetramer. The number of M2-specific CD8 T cells in spleen was significantly increased ($p < 0.05$) in all infected animals compared to the uninfected control, regardless of prior vaccination (Fig.4.5A). Also, there was no increase in the total number of splenic $CD8^+$ T cells following challenge (Fig.4.5C). In contrast, there was a significant increase in the number of M2-specific $CD8^+$ T cells in the lungs of the nanopariticle

vaccinated animals following challenge (Fig.4.5B), and this increase was not due to a general increase in the total pulmonary $CD8^+$ T cell population (Fig.4.5D). Overall, these data indicate that vaccination with RSV G protein-based nanoparticles before RSV infection significantly increases the number of RSV M2-specific $CD8^+$ T cells in the lungs post-challenge, suggesting that the immune response against the RSV G CX3C motif prevents G protein modulation of adaptive immune responses to RSV infection, permitting a more potent Th1 and $CD8^+$ T lymphocyte responses to RSV M2 at the site of viral infection.

Mice vaccinated with RSV G nanoparticles are protected against RSV disease

To determine if the increase in M2-specific $CD8^+$ T cells in lungs was indicative of pulmonary inflammation and disease, we evaluated other hallmarks of RSV enhanced disease. Weight loss is used as a marker of pathology and was measured from day of viral challenge. Unvaccinated control mice lost weight immediately, peaking at ~18% on day 4 post challenge, while mice vaccinated with GA2, GCH17 or GB1 experienced milder weight loss peaking at ~7% on day 3 post challenge (Fig.4.6A). The vaccinated mice also began to regain the lost weight more quickly than the unvaccinated mice, suggesting that vaccination induced potent memory immune responses that efficiently cleared the RSV infection. Previous studies have shown that the magnitude of weight loss correlates with cell recruitment to the lung compartment. In order to evaluate whether RSV G protein nanoparticle vaccination induces pulmonary cell recruitment, we analyzed the lungs from infected mice and naïve mice at day 5 post challenge by flow cytometry. The nanoparticle vaccinated mice showed no increase in the number of eosinophils (Fig.4.6B) or neutrophils (Fig.4.6C) compared to the infected unvaccinated control or to the uninfected mice, demonstrating that vaccination with G

nanoparticles does not prime for either lung eosinophilia or neutrophilia upon infection. On the other hand, we detected an increase in the number of macrophages in vaccinated animals compared to the infected unvaccinated controls, but this difference was only statistically significant ($p = 0.016$) for the GCH17 vaccinated group (Fig.4.6D). Macrophages play a key role during RSV infection, and it has been shown that the absence of lung macrophages dampens the innate response to RSV infection and increases the peak viral load, but does not modify weight loss or lung function (64). It is possible that the increased recruitment of macrophages is a result of the elevated number of M2-specific $CD8^+$ T cells and Th1 cells secreting IFNγ in the lungs of vaccinated animals and that the increase in macrophages contributes to the elimination of RSV infected cells and subsequent reduction in the pulmonary viral load (Fig.4.3).

Discussion

It is well established that both humoral and cellular immunities play important roles in host's defense against RSV infection (2, 3, 12). In general, evidences indicate that neutralizing antibodies are the major factors conferring protection against RSV infection, while cell-mediated responses are of greater importance for virus clearance (18). Among RSV viral antigens, the F protein has been shown to induce neutralizing antibodies and protective immunity in humans and in various animal models (20, 21, 49, 68, 73). RSV G protein has also been shown to induce neutralizing antibodies and protective immunity, but in lesser efficiency compared to the F protein (8, 34, 35, 45, 62). However, mixtures of monoclonal anti-G antibodies that are specific for non-overlapping epitopes within the G protein are highly neutralizing due to their synergistic effect, suggesting that serum polyclonal anti-G antibodies can effectively neutralize RSV via creating steric hindrance that inhibit binding of the invading virions to the host cell membrane

(42). Nevertheless, RSV G protein also functions to enhance RSV disease pathogenesis (54, 75, 76, 78). Previous studies have reported various immuno-modulatory roles of RSV G protein that are involved in the dysregulation of host innate and adaptive immune responses (33, 77, 79, 81), and one of the mechanisms that G protein utilizes is the CX3C chemokine mimicry (78). The CX3C chemokine mimicry is attributed to the central conserved region of the G protein which contains four cysteine residues (1, 33, 40, 58, 78), as this region is homologous to the receptor binding region of fractalkine and has been shown to possess fractalkine-like leukocyte chemotactic activity (78). As such, RSV G protein functions to negatively modulate the host immune response to RSV infection by inhibiting fractalkine-mediated trafficking of CX3CR1+ immune cells (33, 76). Interestingly, vaccination to induce anti-G antibodies that are specific to the central conserved region of the G protein and inhibit G protein interaction with the fractalkine receptor, CX3CR1, have been shown to reduce parameters of RSV disease, including weight loss, pulmonary inflammation, and lung virus titer (94). Such findings suggest that RSV G polypeptide vaccination approach to generate antibodies specific to the central conserved region of the G protein that block RSV G protein-CX3CR1 interaction may be an effective strategy in developing safe and effective RSV vaccine that prevent RSV G protein-mediated immune evasion and disease pathogenesis (94). Additionally, particulate vaccines have been shown to induce potent immune responses in the absence of conventional adjuvants due to the recognition by immune cells of the particle structures, which mimic natural pathogens such as viruses and bacteria. By incorporating well-defined antigenic epitopes in micro- and nanoparticle constructs, investigators have demonstrated improved immunogenicity of both B and T cell epitopes in a number of model systems.

In the present study, we demonstrated that vaccination with LbL nanoparticle vaccines comprising the conserved RSV G protein CX3C motif of RSV/A2, /B1 or /NY/CH17/93 strains induces strong neutralizing antibody responses that also inhibited RSV replication *in vivo* following live virus challenge. Importantly, our study also demonstrates that vaccination with RSV/B1 and NY/CH17/93-derived nanoparticles induced cross-protection against RSV/A2 infection. It has been suggested that an efficacious RSV vaccine should confer protective immunity against both RSV A and RSV B, the two circulating RSV strains. It has been suggested that an infection with RSV A strains may exhibit more severe disease symptoms compared to an infection with RSV B stain (10, 35). However, both strains circulate together during RSV epidemics increasing the RSV antigenic heterogeneity and contributing to reinfection (10, 35). Also, shifting in the predominance of circulating RSV strains occurs in 1- or 2- year cycles (90). Therefore, future experiments will investigate whether the antibodies generated by vaccination with GA2, GCH17 or GB1 nanoparticles could also provide protection against RSV/B1 infection.

Although antibody responses are crucial for protection against RSV infection, T cell-mediated responses play an important role in virus clearance. In the present study, we demonstrated that vaccination with the RSV G nanoparticles induces potent $CD8^+$ T cell responses and balanced Th1/Th2 responses. Previous studies have shown that efficient virus clearance requires effective Th1 response characterized by IFNγ, IL-2 and IL-12 secretion and that a bias toward Th2 response can contribute to the sever RSV disease characterized by airway hyperresponsiveness, mucus over-production, whizzing, and pulmonary eosinophilia (6, 9, 54). Similarly, it has been reported that $CD8^+$ T cells are essential in the clearance of RSV, and that virus clearance is closely associated with an increase of RSV-specific $CD8^+$ cytotoxic T

lymphocyte activity in the lungs (72). Consistent with these reports, we observed a correlation between the nanoparticle vaccination-induced cellular responses (increased in IFNγ and IL-4 secreting cells and increased numbers of M2-specific $CD8^+$ T cells) and the reduction in RSV replication in the lungs of the challenged animals. By contrast, our unvaccinated control mice (that received PBS) developed less potent T cell responses and exhibited increased viral replication in the lungs. We also observed an increase in the frequency of alveolar macrophages in the G nanoparticle vaccinated animals, without displaying any apparent pulmonary eosinophila or neutrophilia, or any significant weight loss after the virus challenge. Previous studies indicate that alveolar macrophages serve as a significant source of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-8, following RSV infection (5, 64), and that depletion of alveolar macrophages is associated with enhanced RSV titers in the lung while showing no significant affects on the T lymphocyte recruitment, weight loss, and overall lung function (64). Additionally, another study has shown that the deficiency of alveolar macrophages in NZB mice were central to the enhanced disease. The authors in this study observed that the depletion of alveolar macrophages in BALB/c mice prior to RSV challenge resulted in airway occlusion and a similar pathology was observed in macrophage deficient NZB mice, suggesting that macrophages may play an important role in restricting the lung viral load and in clearing apoptotic cellular debris which otherwise can promote severe RSV-related lung disease (67). These finding underscores that alveolar macrophages contribute to the elimination of RSV but not to pulmonary inflammation. Accordingly, our results suggest that RSV G nanoparticle vaccination induces potent cell-mediated immune responses that contribute to the clearance of RSV infection and suppress infection-mediated disease pathogenesis.

Interestingly, we observed that priming of mice with RSV G nanoparticle vaccines induced an increase in the number of M2-specific $CD8^+$ T cells in lungs upon live RSV challenge, although none of the nanoparticle vaccines contained an RSV M2 epitope. One possible explanation for such observation is that RSV G nanoparticle vaccination may have generated anti-G antibodies specific to the central conserved region that effectively prevented the binding of RSV G protein to CX3CR1 expressed on leukocytes, consequently inhibiting the natural immuno-modulatory effect of the RSV G protein that interferes with the $CD8^+$ T cell recruitment and their cytotoxic function in the infected lungs. Accordingly, studies have shown that $CD8^+$ cells expressing CX3CR1 are a major component of the cytotoxic response to RSV infection, and that infection with an RSV mutant lacking the G gene dramatically increases the number of $CX3CR1^+$ T cells in the lungs and reduces Th2-type cytokine expression (54, 80). Another possible explanation is that RSV G-specific memory $CD4^+$ T cells induced during the vaccination regimen may have responded rapidly upon G-antigen exposure following RSV challenge, resulting in the rapid secretion of Th1 cytokines and chemokines that contributed to the increased activation and proliferation of naive $CD8^+$ T cells and their subsequent recruitment to the lungs. A third explanation and probably the most obvious would be that RSV G and M2 proteins shared a common T cell epitope, but this is less unlikely since $CD4^+$ and $CD8^+$ T cells from mice vaccinated with RSV G protein nanoparticles do not respond to stimulation with $M2_{82\text{-}90}$ in the absence of virus challenge (data not shown). Further study is required to characterize and understand the specific role of anti-G immunity on M2-specific $CD8^+$ T cell responses.

Overall, our data show that vaccination of BALB/c mice with the RSV G nanoparticle vaccines is associated with the induction of neutralizing antibody response, increase in RSV G-

and M2- specific T cell response following RSV A2 challenge, and reduction in RSV disease pathogenesis. Taken together, our findings demonstrate that the vaccination approach using LbL nanoparticles engineered to carry the RSV G protein CX3C motif may be a promising strategy in developing a novel RSV vaccine that is effective in preventing RSV G protein-mediated immune modulation and disease pathogenesis.

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TABLE 4.1. Designed peptides carrying the CX3C chemokine epitope[a] of RSV G protein.

Name	Epitope	Peptide sequence	RSV strain
GA2	169-198	NFVPCSICS NNPTCWAICKRIPNKKPGKKTK$_{20}$Y	A2
GCH17	170-198	FVPCSICS NNPTCWDICKRIP S KKPGKKT K$_{20}$Y	NY/CH17/93
GB1	170-198	FVPCSICGNNQLCK S ICKT IPS N KPKKK PK$_{20}$Y	B1

[a] The location of the CX3C motif in the G protein is underlined.

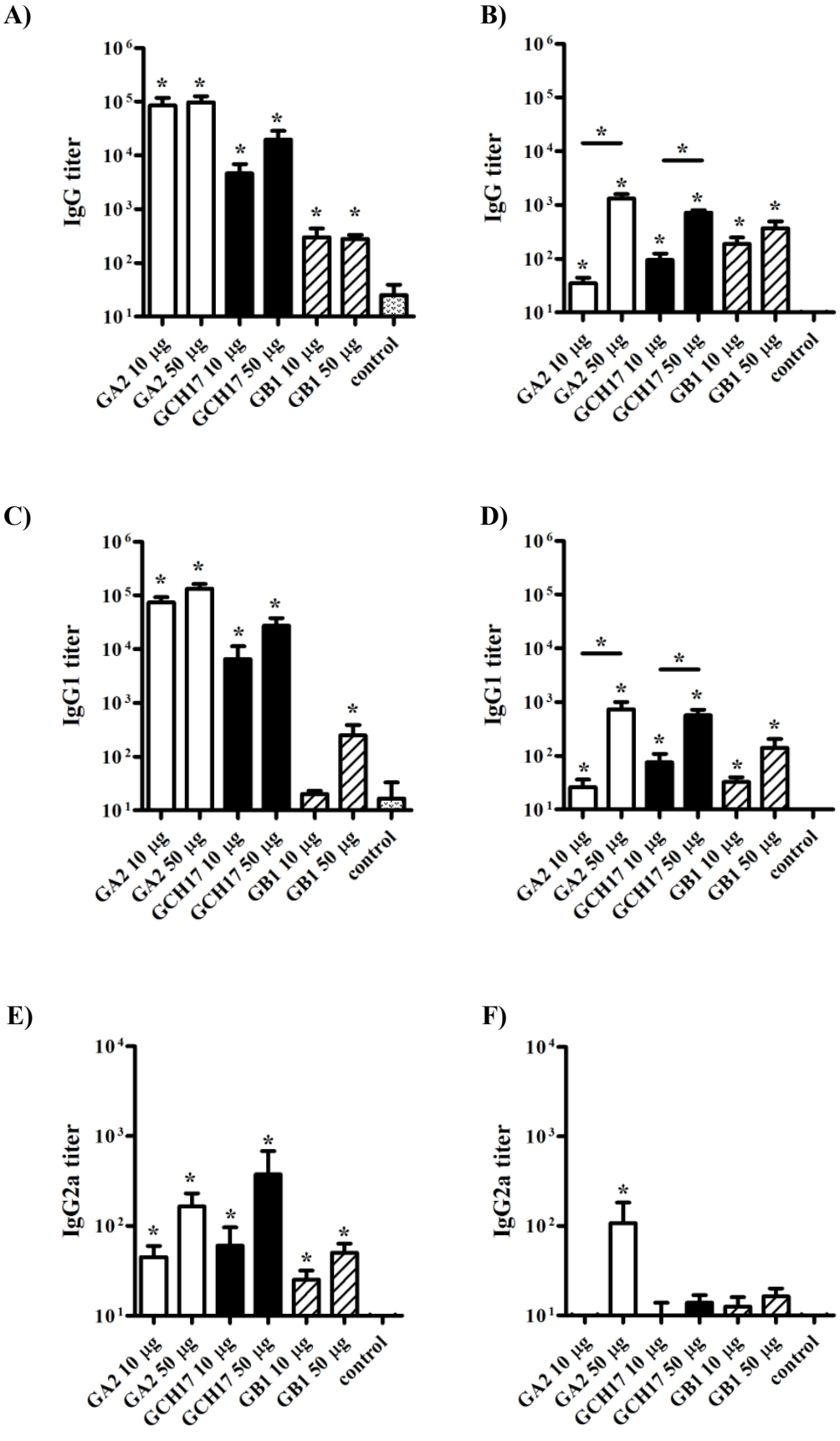

A)
B)
C)
D)
E)
F)
IgG titer
IgG1 titer
IgG2a titer
GA2 10 µg
GA2 50 µg
GCH17 10 µg
GCH17 50 µg
GB1 10 µg
GB1 50 µg
control

FIGURE 4.1. Antibody responses elicited by vaccination with RSV G nanoparticles. Groups of BALB/c mice were inoculated s.c. with nanoparticle diluted in PBS to yield 10 μg or 50 μg of designed peptide per dose. Sera were obtained from blood taken 21 days after the secondary inoculation. RSV/A2 (A, C, and E) and RSV/B1 (B, D, and F)-specific IgG (A and B), IgG1 (C and D) and IgG2a (E and F) levels were determined by indirect ELISA. Bars represent the average titer of each group with error bars representing the SEM from n=5 mice per group. * = $p<0.05$, significant difference as determined by Student's *t* test compared with PBS vaccinated control mice (control) or comparison between dose levels of the same vaccine.

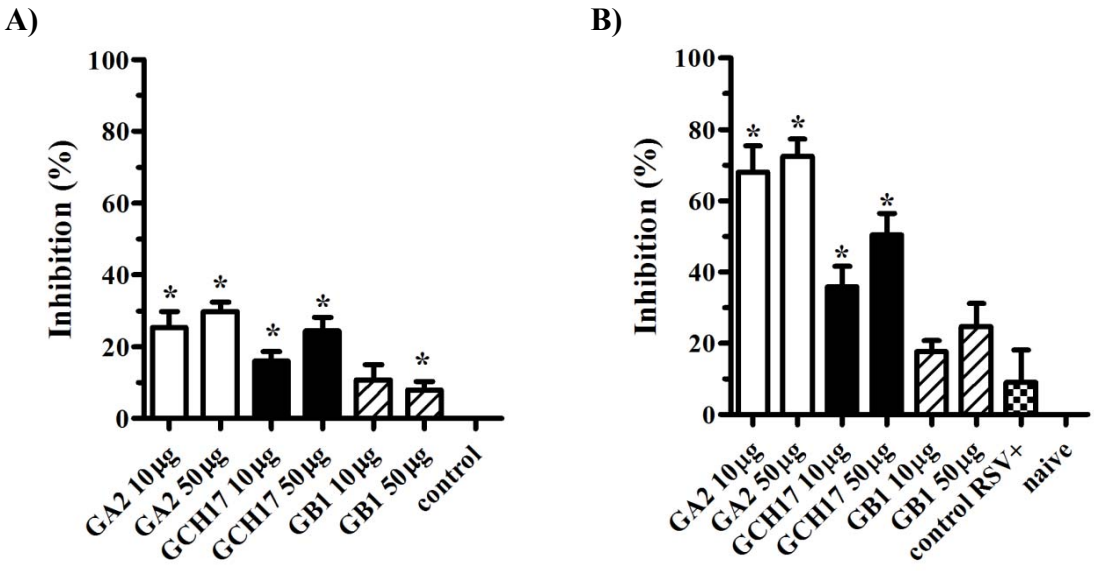

A)
B)
Inhibition (%)
100
80
60
40
20
0
GA2 10µg
GA2 50µg
GCH17 10µg
GCH17 50µg
GB1 10µg
GB1 50µg
control
control RSV+
naive

FIGURE 4.2. RSV/A2 neutralizing antibodies in mice vaccinated with RSV G nanoparticles. Sera from vaccinated mice were evaluated for neutralizing antibodies by plaque inhibition assay. Serum samples were collected 21 days after the secondary inoculation (A) and 5 days post challenge with 10^6 PFU of RSV/A2 (B). A representative assay using a 1:40 serum dilution is shown, and data are presented as the percent RSV plaque inhibition calculated from the average of triplicate sample wells and expressed as the percent plaque inhibition relative to control sera obtained from PBS vaccinated mice. Error bar represents the SEM from n=5 mice per group. * = $p<0.05$, significant difference compared with PBS vaccinated control (control) or PBS vaccinated control mice infected with RSV/A2 (control RSV +) mice as determined by Student's *t* test.

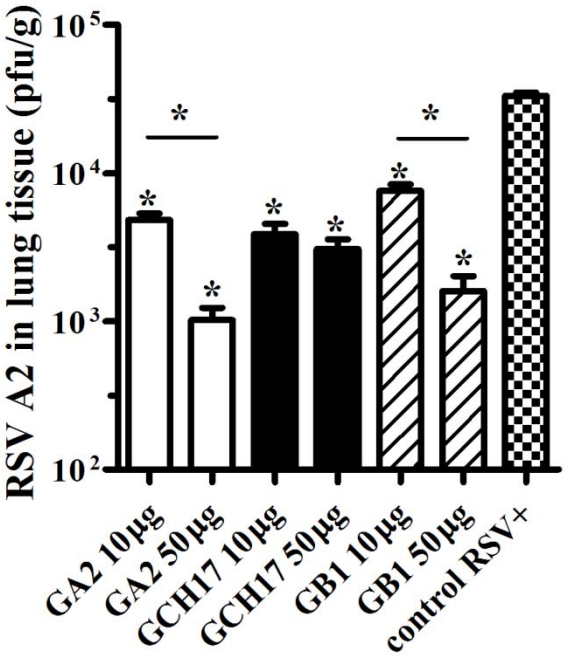

RSV A2 in lung tissue (pfu/g)
10^5
10^4
10^3
10^2
*
*
*
*
*
*
*
GA2 10µg
GA2 50µg
GCH17 10µg
GCH17 50µg
GB1 10µg
GB1 50µg
control RSV+

FIGURE 4.3. Lung virus titers following RSV/A2 challenge of vaccinated mice. BALB/c mice were vaccinated s.c. with 10 μg or 50 μg doses of nanoparticles (GA2, CH17 and GB1) on days 0 and 21, and challenged i.n. on day 42 with 10^6 PFU of RSV/A2. Lung virus titers were determined 5 days post-challenge by the immunostaining plaque assay (79). The data are presented as PFU/g of lung tissue. Error bar represents the SEM from n=5 mice per group. * = $p<0.05$, significant difference as determined by Student's *t* test by comparison with PBS vaccinated control mice infected with RSV/A2 (control RSV+) or comparison between dose levels of the same vaccine.

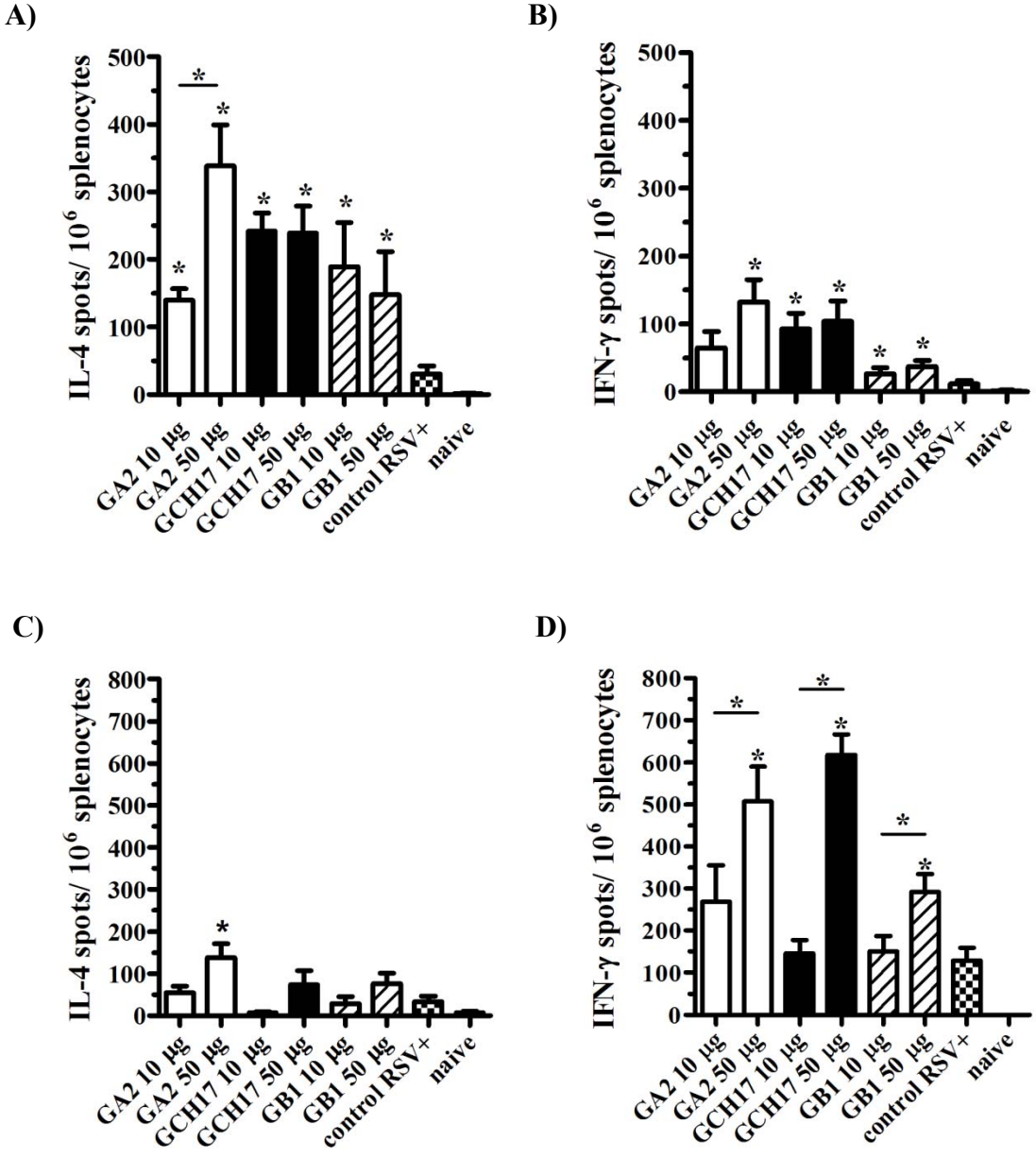

A)
B)
C)
D)
IL-4 spots/ 10^6 splenocytes
IFN-γ spots/ 10^6 splenocytes
0
100
200
300
400
500
600
700
800
GA2 10 µg
GA2 50 µg
GCH17 10 µg
GCH17 50 µg
GB1 10 µg
GB1 50 µg
control RSV+
naive
*

FIGURE 4.4. Frequency of RSV-specific IFNγ and IL-4 secreting cells after virus challenge. The number of $G_{183\text{-}197}$ specific IL-4 (A) or IFNγ (B) producing splenocytes and $M2_{82\text{-}90}$ specific IL-4 (C) or IFNγ (D) producing splenocytes was determined by ELISPOT in cells harvested 5 days post-challenge. The data are presented as cytokine spots/10^6 splenocytes. Error bars represent the SEM from n=5 mice per group. * = $p<0.05$, significant difference as determined by Student's *t* test by comparison with PBS vaccinated control mice infected with RSV/A2 or comparison between dose levels of the same vaccine.

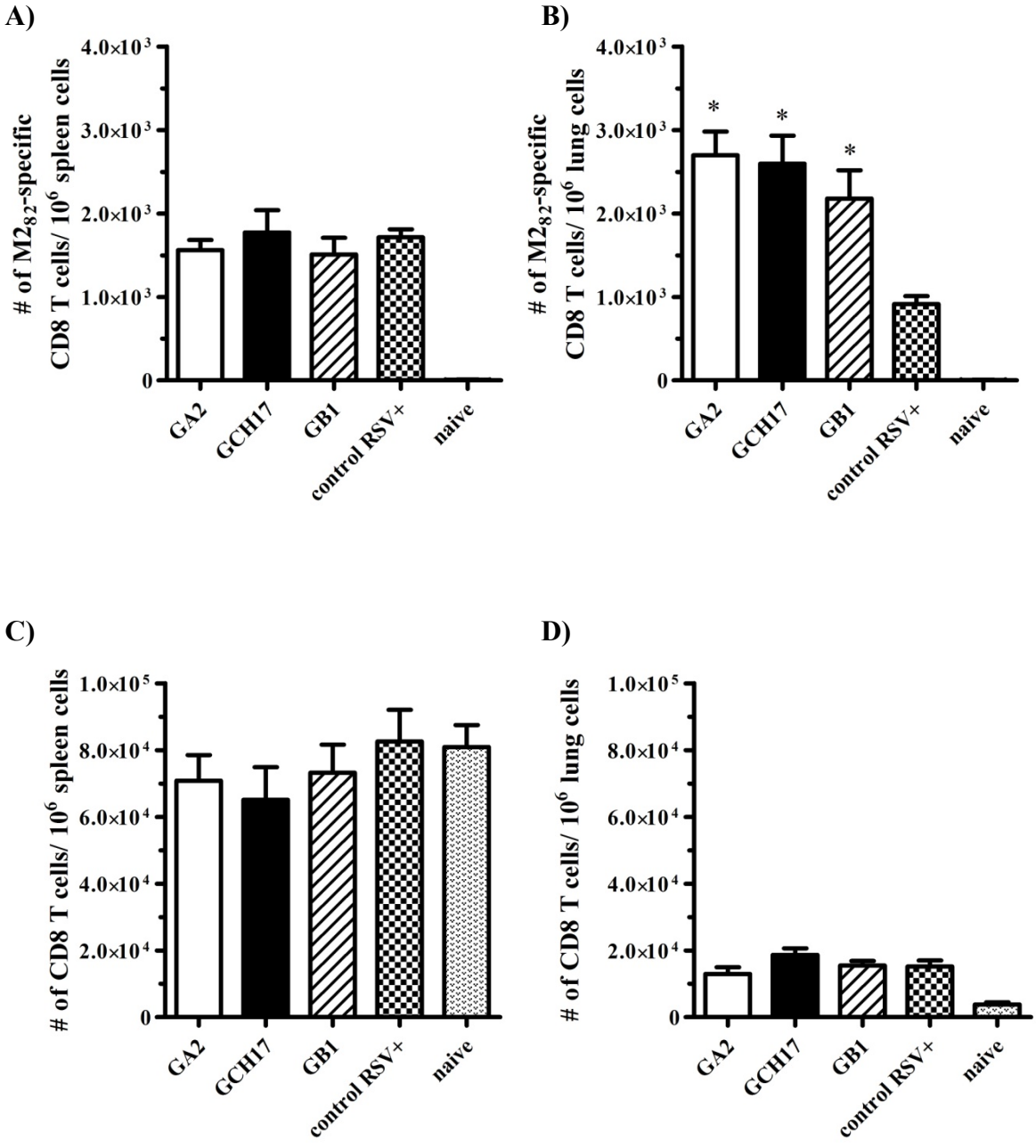
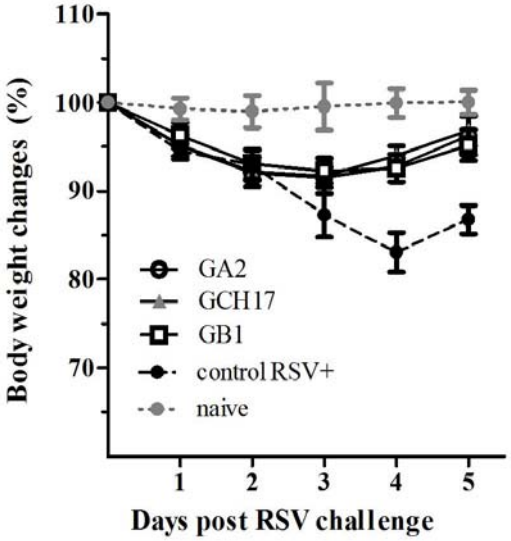
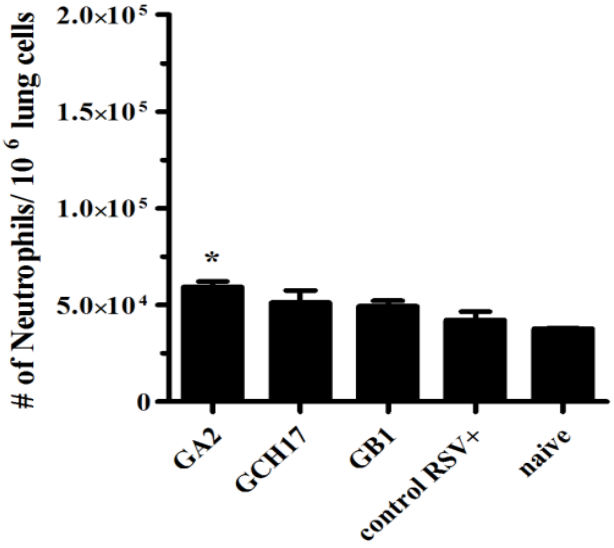

A)
# of M2$_{82}$-specific CD8 T cells/ 10^6 spleen cells
4.0×10^3
3.0×10^3
2.0×10^3
1.0×10^3
0
GA2
GCH17
GB1
control RSV+
naive
B)
# of M2$_{82}$-specific CD8 T cells/ 10^6 lung cells
4.0×10^3
3.0×10^3
2.0×10^3
1.0×10^3
0
*
*
*
GA2
GCH17
GB1
control RSV+
naive
C)
# of CD8 T cells/ 10^6 spleen cells
1.0×10^5
8.0×10^4
6.0×10^4
4.0×10^4
2.0×10^4
0
GA2
GCH17
GB1
control RSV+
naive
D)
# of CD8 T cells/ 10^6 lung cells
1.0×10^5
8.0×10^4
6.0×10^4
4.0×10^4
2.0×10^4
0
GA2
GCH17
GB1
control RSV+
naive

FIGURE 4.5. Enumeration of RSV M2-specific $CD8^+$ T cells using MHC class I tetramer. Spleen (A) and lung (B) cell suspensions obtained from mice challenged with RSV/A2 were stained with APC-labeled M2-specific H-$2K^d$ tetramer and anti-mouse CD8- IgG-PerCP conjugated. The number of splenic (C) and pulmonary (D) CD8 T cells per 10^6 events was determined by flow cytometric analysis. Error bar represents the SEM from n = 5 mice per group. * = $p<0.05$, significant difference compared with PBS vaccinated control mice infected with RSV/A2, as determined by Student's *t* test.

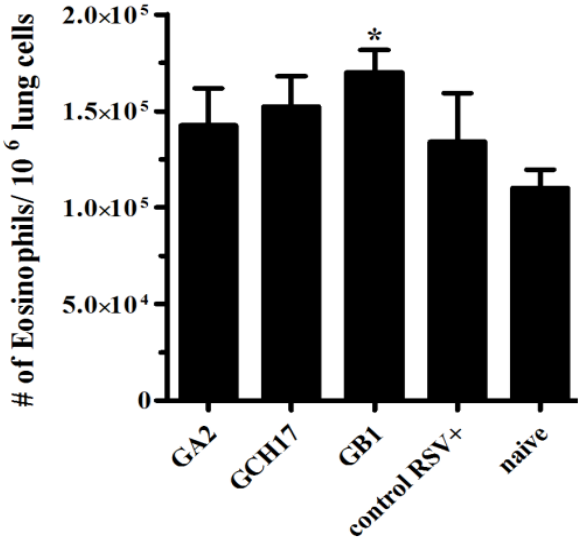
A)


Body weight changes (%)
110
100
90
80
70
GA2
GCH17
GB1
control RSV+
naive
1
2
3
4
5
Days post RSV challenge

B)


# of Neutrophils/ 10 6 lung cells
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
*
GA2
GCH17
GB1
control RSV+
naive

C)


# of Eosinophils/ 10 6 lung cells
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
*
GA2
GCH17
GB1
control RSV+
naive

D)

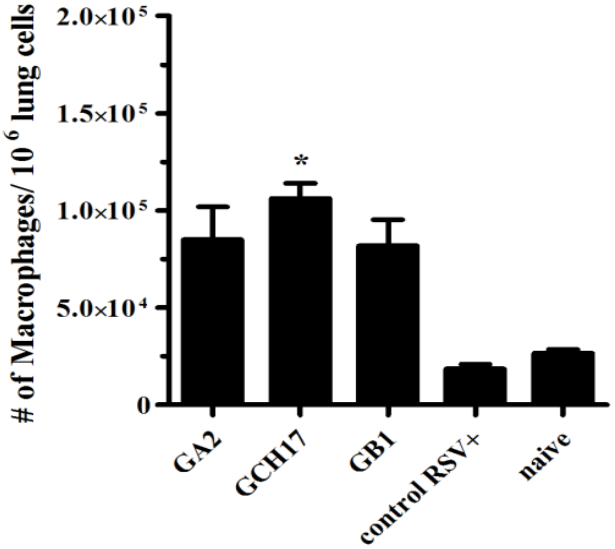

# of Macrophages/ 10 6 lung cells
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
*
GA2
GCH17
GB1
control RSV+
naive

FIGURE 4.6. Weight loss and pulmonary cell recruitment upon RSV infection. Mice were vaccinated with 50 μg of RSV G nanoparticles and challenged at 6 weeks post boost with 10^6 PFU of RSV/A2. Animals were weighed daily and percentage of weight loss calculated based on day 0 (A). Lungs were harvested at day 5 post challenge and cell suspension were immunolabeled with anti-Ly6G, anti-Ly6C, anti-CD125 and anti-siglecF, and examined by flow cytometry. Based on surface markers expression, three populations were identified: neutrophils [$Ly6G^{high}$ $Ly6C^{int}$ $CD125^{low}SiglecF^{-}$] (B), eosinophils [$Ly6G^{int}$ $Ly6C^{high}$ $CD125^{high}$ $SiglecF^{+}$] (C), and macrophages [$Ly6G^{int}$ $Ly6C^{int}$ $CD125^{low}SiglecF^{+}$] (D). The data are presented as the total number of cells/10^6 lung cells. Values represent mean ± SEM of cells per lung (n=5). * = $p<0.05$, significant difference compared with PBS vaccinated control mice infected with RSV/A2, as determined by Student's *t* test.

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CHAPTER 5

CONCLUSION

In the first study, we examined a vaccine strategy using RSV G protein polypeptide vaccination to generate IgG antibodies reactive to the central conserved region of the RSV G protein as a strategy block the interaction of the RSV G protein CX3C motif from RSV strain A2 or B1 with the fractalkine receptor, CX3CR1. We hypothesized that RSV G protein blocking antibodies would inhibit binding of RSV A and RSV B G proteins to CX3CR1 and provide heterosubtypic protection, thereby preventing RSV G protein-induced immune modulation and reducing disease pathogenesis. Accordingly, our results show that antibodies specific to the central conserved cysteine loop region of the RSV G protein block binding of the RSV A2 and RSV B1 native G protein to CX3CR1 expressed by 293 cells compared to the antibodies specific to N- and C- terminal hypervariable regions of the RSV G protein. The importance of these findings are that RSV G polypeptide vaccination can induce antibodies that recognize central conserved G protein peptide sequences shared by RSV A2 and B1 strains, and the reactivity of these antibodies can inhibit G protein binding to CX3CR1 and virus replication, an aspect which has not been previously described previously and which is critical in RSV vaccine development.

In our second study, we adapted layer-by-layer (LbL) nanoparticle technology to produce synthetic nanoparticles carrying one of the three designed target polypeptides comprising the CX3C motif of the RSV G protein from RSV A2, B1 or NY/CH17/83 strains. Particulate vaccines have been shown to induce potent immune responses in the absence of conventional

adjuvants due to several mechanisms including efficient phagocytosis of these particles, cross-presentation, and activation of dendritic cells by increased cytokine production and co-stimulatory marker expression. By incorporating well-defined antigenic epitopes in micro- or nanoparticle constructs, it is possible to achieve increased potency of the nanoparticle vaccine constructs. We hypothesized that vaccination with LbL nanoparticles expressing RSV G polypeptides spanning the CX3C motif would provide for optimal induction of antibody responses in a safe and effective RSV vaccine. Our data show that vaccination of BALB/c mice with the RSV G nanoparticle vaccines is associated with the induction of neutralizing antibody response, increase in RSV G- and M2- specific T cell response following RSV A2 challenge, and reduction in RSV disease pathogenesis. Taken together, our findings demonstrate that the vaccination approach using LbL nanoparticles engineered to carry the RSV G protein CX3C motif is a good strategy for developing efficacious RSV vaccines that prevent RSV G protein-mediated immune modulation and disease pathogenesis.