RICE UNIVERSITY

Functional and Structural Studies of Respiratory Syncytial Virus Fusion (RSV F) Protein and Neutralizing Antibody

by

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ABSTRACT

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Human respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract infections in newborns, young children, and the elderly; causing significant morbidity and mortality each year. Unfortunately, there is currently no licensed vaccine for prophylaxis of RSV infection, and the therapeutic options are limited. The research and development of efficient RSV vaccine or therapeutic antibody has been a priority globally.

RSV fusion (F) glycoprotein on the surface of the particle plays a central role in infection, mediating viral entry into host. It is an almost exclusive target for vaccine design and therapeutic antibody development in this field. RSV F has multiple antigenic sites on the pre-fusion and the post-fusion conformations. In Chapter 2, we characterized a series of monoclonal antibodies. We found a novel one, designated as R4.C6, binds with sub-nanomole affinity to a unique neutralizing site that occupies an intermediate
position between antigenic sites II and IV on the globular head. Cryo-EM and 3D image reconstruction at 3.9 Å resolution was used to reveal the interactions of R4.C6 Fab in complex with the RSV F glycoprotein. Three R4.C6 Fab were found to bind to a quaternary epitope across two protomers; both heavy chain and light chain have interactions with site II of one protomer and site IV of the neighboring protomer. These results further our understanding of the antigenic complexity of the F protein and provide new insight into the design of RSV vaccines.

The F glycoprotein is structurally complex with multiple conformations. It undergoes conformation shift from prefusion to pre-hairpin intermediate and finally to postfusion state. The combined energies released during multiple conformational rearrangements are used to bring the N- and C-terminal of RSV F1 subunit together, forcing the close contacts of virus and host cell membranes, and finally membrane fusion. In Chapter 3, we performed single particle cryo-EM analysis for another form of RSV F, named as BV2052. The 5 Å resolution density map shows some features that distinguished from the prefusion and postfusion state. BV2052 is in an intermediate state. This helps us understand the fusion mechanism.
The research present here is accomplished with the support and help from many people, including my advisor, professors, colleagues, collaborators, friends, funding agencies and my families. I am deeply grateful for them.

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Chapter 1

Introduction

1.1 Paramyxoviruses

Paramyxoviruses are a family of viruses whose genome contains monopartite, negative-sense, single-stranded RNA. There are three genera in this virus family: Paramyxovirus, Pneumovirus, and Morbillivirus. Paramyxovirus includes the parainfluenza viruses and mumps virus. Pneumovirus includes respiratory syncytial virus (RSV) and human metapneumovirus (hMPV). Morbillivirus includes the measles virus. The paramyxoviruses can infect a wide range of hosts, including humans, vertebrates, and birds. This family of viruses can cause influenza-like symptoms. But they have some unique features different from the orthomyxoviruses. The differences between paramyxoviruses and orthomyxoviruses are listed in Table 1.1 (1). This thesis mainly focuses on respiratory syncytial virus, although the other paramyxoviruses are also important and may have similar characteristics in terms of morphology, genome composition, virion structure, and replication cycle.
<table>
<thead>
<tr>
<th></th>
<th>Paramyxoviruses</th>
<th>Orthomyxoviruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classified into</td>
<td>Parainfluenza, mumps, measles, and respiratory syncytium virus</td>
<td>Influenza viruses and other non-human viruses</td>
</tr>
<tr>
<td>Particle size</td>
<td>120-300 nm (somewhat pleomorphic)</td>
<td>80-120 nm (highly pleomorphic)</td>
</tr>
<tr>
<td>RNA genome</td>
<td>Non-segmented</td>
<td>Segmented</td>
</tr>
<tr>
<td>Replication</td>
<td>In cytoplasm</td>
<td>In nuclear</td>
</tr>
<tr>
<td>Syncytial formation</td>
<td>Yes (F protein functions at normal physiology pH)</td>
<td>No (HA functions at acid pH)</td>
</tr>
<tr>
<td>Surface spikes</td>
<td>Hemagglutinin (H) and Neuraminidase (N) in same spikes</td>
<td>Hemagglutinin (H) and Neuraminidase (N) in different spikes</td>
</tr>
<tr>
<td></td>
<td>Parainfluenza virus has both H and N activities; Measles lacks N activity, and RSV lacks both H and N activities</td>
<td></td>
</tr>
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Table 1.1: Comparison of Paramyxoviruses family and Orthomyxoviruses family. Some of the most important characteristics of Paramyxoviruses family and Orthomyxoviruses family are compared.

1.2 Respiratory syncytial virus (RSV)

1.2.1 Discovery of respiratory syncytial virus

Respiratory syncytial virus (RSV) is a member of paramyxoviridae family. It was firstly identified in 1956 from upper airway secretion of chimpanzee monkeys with bronchiolitis symptoms and characterized as chimpanzee coryza agent. During the same time, a previously unidentified virus was also isolated from infants with lower airway infection. Later, these viruses were renamed as ‘respiratory syncytial virus’ (2). Ever since its discovery, RSV has become recognized as a leading cause of global infectious
disease morbidity and mortality, especially among infants, young children, and elderly people.

1.2.2 Respiratory syncytial virus morbidity and mortality

RSV can cause mild cold-like symptoms, such as runny nose, decrease in appetite, coughing, sneezing, fever, and wheezing. Furthermore, RSV can cause more severe infections as bronchiolitis and pneumonia. It is the leading cause of bronchiolitis and pneumonia diseases in newborn, young children and adults over 65 years old. Premature infants, young children and elderly people with immune system deficiency are more susceptible to get RSV-induced severe acute lower respiratory tract infection. Almost all children will have RSV infection during the first two years of their life. Antibodies against RSV were detected in more than 90% of 2-years children, indicating the infection of RSV happened at some time (3).

RSV causes significant morbidity and mortality. Worldwide, RSV is one of the predominant causes of death in children. Approximately 66,000-199,000 children aged younger than 5 years died with RSV-related acute lower respiratory tract infection in 2005, and this mortality number is 59,600 (48,000–74,500) in 2015 (4,5). A large proportion of the death has at least one comorbidity. As for the adults older than 65 years, it is estimated 14,000 deaths would be caused by RSV infection annually (6). RSV may cause even higher mortality rate in some particular populations, such as the organ transplant recipients.
1.2.3 Respiratory syncytial virus classification

Human respiratory syncytial virus has two subtypes, type A and type B. This classification is initially based on monoclonal antibodies attachment analysis against surface proteins and other structural proteins of hRSV. The analysis also leads to the conclusion that type A and type B have evolved separately (7). Since the virus surface attachment glycoprotein (G protein) is the most variable gene product, antigenic reaction by using monoclonal antibodies specific against the G protein was used to classify hRSV (8). Nowadays, genetic analyses were used to classify hRSV into group A and group B. Based on the genotypes there are at least 12 RSV-A (ON1, GA1 to GA7, SAA1, NA1, NA2 and CB-A) and 22 RSV-B (GB1 to GB4, SAB1 to SAB3, BA1 to BA12, URU1, URU2, and CB-B) genotypes (9). Besides that, some novel genotype viruses keep emerging in recent years.

During each seasonal epidemic, several strains of both subtype A and subtype B co-circulate and differ across worldwide. Compared with RSV-B, RSV-A is less common, but it may be more harmful, as there were more RSV-A positive patients detected in some studies (10).

1.2.4 Respiratory syncytial virus biology

Human respiratory syncytial virus (hRSV) is a negative sense, single-stranded RNA virus, belonging to the *Paramyxoviridae* family. The 15.2 kilobase (kb) hRSV genome includes a short 3’ leader region, a 5’ trailer region and 10 viral genes in between, including NS1, NS2, N, M, P, G, F, SH, M2 and L genes. Each gene encodes a
single viral protein, except M2 genes, which encodes two distinct proteins M2-1 and M2-2 (11,12). *(Figure 1.1)*

![Figure 1.1: Schematics of hRSV genome.](image)

RSV genome is depicted 3’ to 5’ showing the 3’ leader, 10 viral genes, which encode 11 viral proteins, and 5’ trailer. The M2 gene has slight overlap with the L gene. The amino acid number of protein encoded by each gene is list underneath.

The basic structure of RSV virion is schematically shown in Figure 1.2. Under the electron microscope, the particles of RSV are observed as spherical, long filamentous, or a combination of the two, ranging from 100 nm to 1,000 nm in diameter (13). The RSV nucleocapsid is packaged into a bilayer lipid envelope, which derived from the host cell plasma membrane. Among the 11 proteins encoded by RSV genome, two (NS1 and NS2) are non-structural proteins and play roles in immune response. The other structural proteins include three transmembrane surface glycoproteins, one inner envelope protein, and five nucleocapsid/polymerase proteins. The large glycoprotein G, the fusion protein F, and the small hydrophobic SH protein are the three surface proteins. They bulge out from the membrane, form separate oligomers and appear as short surface spikes. The RSV F and G glycoproteins are viral neutralization antigens and also the major protective antigens. The matrix M protein is the only one inner envelope protein and is essential for particle assembly. It is nonglycosylated and forms a proteinous layer underneath the lipid
bilayer envelope. The five nucleocapsid/polymerase proteins are the nucleoprotein (N), the phosphoprotein (P), the transcription proteins (M2-1 and M2-2), and the large polymerase subunit (L). Nucleoprotein N is tightly bound to the genomic RNA forming the left-handed helix structure. Phosphoprotein P binds N and L; it forms the RNA-dependent RNA polymerase (RdRp) together with L. M2-1 acts as RdRp co-factor, regulating the transcription of viral mRNAs. These nucleocapsid proteins are essential for viral RNA transcription and replication (14,15).

Figure 1.2: Schematic representation of hRSV virion with the locations of viral proteins. The G, F, and SH proteins are present in the surface as glycoprotein spikes. The M protein underlies the lipid bilayer envelope. The N, P, L, and M2-1 proteins are packed with RNA to form the ribonucleocapsid.
NS1 and NS2 proteins

Non-structural proteins 1 and 2 (NS1 and NS2; 139 and 124 amino acids, respectively) are uniquely present in RSV. The mRNAs transcript NS1 and NS2 are two of the most abundant RNAs in an infected cell. These two proteins play important roles in interfering with immune responses. NS1 and NS2 inhibit the induction of alpha/beta interferon (IFN-α/β) by cooperatively suppressing the activation and nuclear translocation of interferon regulatory factor 3 (IRF-3), as well as by inducing the activation of nuclear factor κB (NF-κB) (16). IFN-α/β is secreted by host cells to defense against pathogens including virus. IRF3 is a transcription factor inducing the expression of IFN-α/β. NS1 and NS2 also inhibit apoptosis of the host cells, thus, prolong the cell life and increase the viral yield (17). The NS1 protein may also interfere with RNA synthesis. It has been suggested as a potent inhibitor of viral RNA transcription and replication by down-regulating the synthesis of viral RNA. The NS2 protein may also act as a transcription inhibitor (18).

M2-1 and M2-2 proteins

The M2-1 and M2-2 proteins are also unique in RSV. Only Ebola virus (EBOV) has a protein named VP30 having the similar function as M2-1 (19). Both M2-1 and M2-2 are translated from a single M2 mRNA. The open reading frames of M2-1 and M2-2 have partial overlap. M2-1 and M2-2 are considered as accessory proteins like NS1 and NS2, but they are important for virus replication. The deletion of these proteins shows detrimental effects to RSV replication (20).
M2-1 is a 194 amino acids protein. As an essential cofactor of the viral RNA polymerase complex, it functions as a transcription elongation and antitermination factor (14,21). M2-1 is a zinc-binding phosphoprotein, with two conformations: phosphorylated and dephosphorylated. The crystal structure of hRSV M2-1 protein indicates its secondary structures are mostly α-helix. M2-1 protomers are known to form a tetramer in disc-like shape; the tetramerization is driven by the forming of a four-helix bundle in the center (22), shown in Figure 1.3. M2-1 protein can be simply divided into four parts: an N-terminal Zinc-binding domain (ZBD), an oligomerization domain, the ‘core’ domain facilitating binding to P protein and RNA, and an unstructural C terminus. The phosphorylation sites are located at Serine58 and Serine61; they are critical for M2-1 antitermination activity (23). RNA-binding is also critical for M2-1 function, as loss of this ability will disable the antitermination activity (24). M2-1’s RNA binding ability is regulated by phospho- and dephospho-state switch.

The 90 amino acid M2-2 protein is encoded by the second ORF in the M2 gene. The translation of M2-2 protein uses a unique mechanism of ribosomal termination-dependent reinitiation (25). M2-2 protein plays an important role in virus infection and pathogenesis, even though it is dispensable for virus replication (26). The high level expression of M2-2 protein will inhibit RSV replication (27). Researches indicate M2-2 involved in regulating the balance between the viral RNA transcription and replication (28).
Figure 1.3: hRSV M2-1 protein function domains and structure.
(A) Function domains of the hRSV M2-1 protein. (B) Crystal structure of hRSV M2-1 protein tetramer in ribbon presentation. (PDB: 4C3D)

Nucleoprotein N

The 391 amino acid N protein is the major protein of viral nucleocapsid. It tightly binds to the viral genome and antigenome to form the helical nucleocapsids, and protect RNA from nuclease digestion. This nucleocapsid serves as the template for viral RNA synthesis. N protein plays an important role in virus assembly and replication. Tawar et al. solved the crystal structure of RSV N protein bound to bacteria RNA (29), shown in Figure 1.4. In this structure, N protein is oligomerized to form a decameric ring representing one turn of the viral helical nucleocapsid. Each N monomer consists of N- and C-terminal domains (NTD and CTD) that are mostly α-helix bundles, and a hinge region linked the two domains. Each monomer interacts with seven nucleotides, with
RNA been buried into the groove formed at the NTD/CTD interface. By using electron tomography, Bakker et al. proves the nucleoprotein-RNA complex nucelocapsid is a left-handed helix (30).

Figure 1.4: Crystal structure of nucleoprotein in ribbon representation. (A) The decameric N ring structure view down the ring axis, with each monomer has one color, and RNA is colored as red. (B) The N protein monomer with N-terminal domain colored as green, and C-terminal domain colored as yellow. RNA is colored as red. (PDB: 2WJ8)

Phosphoprotein P

The phosphoprotein (P), having 241 amino acids, is an essential polymerase cofactor. RSV P protein forms homo-tetramer and interacts with L, N, and M2-1. The P protein is constitutively phosphorylated, and the phosphorylation may affect the protein oligomerization (31). The unphosphorylated P protein expressed in bacteria cannot form the oligomer. RSV P protein is important in both transcription and replication, and the phosphorylation of P protein is required for efficient replication (32). Act as a cofactor, P
helps to stabilize of the protein L and to translocate the polymerase complex along the N-RNA template (33). P is also believed to help to maintain the solubility of newly synthesized RNA-free N protein by forming a soluble complex (N-P complex) with it (34). The C-terminal region of P protein is believed to be essential and necessary for the interactions with N protein (35). Bioinformatic analysis indicates RSV P protein has a central structural domain with two disordered regions on either side (36). Because of this structural disorder, there is so far no available structure for RSV P protein.

Large polymerase L

The RSV large polymerase subunit (L) is the largest RSV protein, with 2165 amino acids. It is the key component of the RNA-dependent RNA polymerase complex and responsible for the primary enzyme activity in transcription and replication. The functions of protein L mainly include catalyzing the transcription of viral mRNAs, capping and polyadenylating each mRNA. Protein L has six conserved regions (CR) with distinct functions and two variable regions (VR) (37). Like as vesicular stomatitis Indiana virus (VSV) polymerase, CRI is responsible for N assembly and P protein binding. CRII is responsible for N-RNA nucleocapsid binding. CRIII has the RNA-dependent RNA polymerase catalytic activity. CRV not only functions in mRNA capping, but also modulates the elongation properties of the polymerase. CRVI serves as the mRNA cap methylase (38-40). Because of the low expression level, large size and low stability, currently there is no crystal structure available for RSV protein L. But there is a high resolution structure of the VSV L protein in complex with a fragment of P protein been
solved using electron cryomicroscopy (41). RSV L protein may have similarity in structure with VSV L protein.

**Matrix protein (M)**

The 256 amino acid matrix protein M is essential for virion morphogenesis. It self-assembles a proteinous layer that underneath the lipid bilayer envelop of virus. M protein initiates the assembly and budding of virus from host cell surface, during which the dimerization of M is required (42). M recruits the RNPs to the plasma membrane for inclusion through the mediating of M:M2-1 interaction. M protein also interacts with the cytoplasmic tails of the G and/or F glycoproteins (43). M protein is dispensable for the formation of viral filaments, but is necessary for the maturation of viral filaments (44).

A unique characteristic of M protein distinct from other RSV proteins is nuclear trafficking. In early stage of infection, M is localized to the nucleus and functions to inhibit the host cell transcription. Later, M is detected in cytoplasmic viral inclusion bodies for virus assembly and budding (45).

Both monomer form and dimeric form M protein were solved by crystallography (42,46). But the biologically active form of M is dimer. The monomer form shows M protein having two compact N- and C- terminal domains linked by an unstructured 36aa linker. The whole structure is composed by mostly \(\beta\)-sheets and some \(\alpha\)-helices (See Figure 1.5).
Figure 1.5: Ribbon representative of the crystal structure of RSV M protein. The N-terminal domain (aa1-126) is colored as slate, and the C-terminal domain (aa 140-255) is colored as red. The linker between these two domains is colored as yellow. (pdb: 2VQP)

Small hydrophobic SH protein

The small hydrophobic SH protein of RSV has 64 amino acids and is a type II transmembrane protein. It is the smallest of the three surface proteins. SH protein has a single α-helical transmembrane domain from amino acid 18 to 43. The 17aa N-terminal domain is the intracellular domain, while the 21aa C-terminal is the extracellular domain (47). SH protein is non-essential for virus replication and entry into host cells. RSV with SH gene deleted is viable as the wild-type virus (48). But it still plays an important role in viral pathogenesis. It was reported SH protein has slight effect on reducing TNF-α induced apoptosis (49). In addition, SH can form homopentamers that function as cation-selective channels and permeabilize membranes (47).
Attachment protein G

The attachment protein G, 298 or 299 amino acids, is the major surface glycoprotein responsible for virus attachment with the host cells (50). G protein is a type II transmembrane protein. The structure of G protein has not been solved yet. It has a N-terminal cytoplasmic domain, a transmembrane domain from aa36-67, an ectodomain comprised of two highly variable but heavily glycosylated mucin-like domains, a central conserved domain (CCD), a cysteine noose domain with four cysteines linked 1-4 and 2-3, a CX3C domain and the following basic heparin-binding domain (HBD) as shown in Figure 1.6 (51). The HBD serves as the attachment site for heparan sulfate (HS) on the surface of host cells (52). G protein uses CX3C domain to bind with the fractalkine receptor CX3CR1. This interaction is important for infection of human airway epithelial cells (53).

G protein is expressed in two forms: membrane-bound (mG) form and secreted (sG) form. sG lacks the 65-74 amino acids at the N-terminus. This secreted form sG acts as an antigen decoy to help inhibit the antibody-mediated antiviral effects (54).

G protein has almost no sequence similarity with other paramyxovirus attachment proteins and no function similarity as haemagglutinin and neuraminidase proteins. It is the most variable protein among RSV strains, with only 53% homology in the amino-acid sequences. Thus, the activity to G protein monoclonal antibodies was used as a basis to classify RSV strains. This characteristic is also helpful in diagnosis.
Figure 1.6: Cartoon schematic of the RSV G protein. The RSV G protein contains a cytoplasmic domain and a transmembrane domain (TM) at the N-terminal, two Mucin-like regions (I & II) and a basic heparin-binding domain. The zoomed schematic shows the central conserved domain (CCD), a CX3C domain, and a cysteine noose domain with four cysteines linked 1-4 and 2-3.

**Fusion Protein F**

The 574 amino acid fusion protein F is the other surface glycoprotein. The primary function of F protein is fusing the viral envelope with the host cell plasma membrane. Both F and G glycoproteins are the target of neutralizing antibodies. Since F protein is the research target in this thesis, more details about F will be discussed in following subchapters.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Locations</th>
<th>Functions</th>
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</thead>
<tbody>
<tr>
<td>NS1 &amp; NS2 Non-structural proteins</td>
<td>Host cell cytoplasm</td>
<td>Suppress innate immune response by inhibit interferon α/β induction and signalling</td>
</tr>
<tr>
<td>N, Nucleocapsid</td>
<td>Nucleocapsid</td>
<td>Encapsidates genomic and antigenomic RNA, component of the RNP template</td>
</tr>
<tr>
<td>P, Phosphoprotein</td>
<td>Nucleocapsid</td>
<td>Essential polymerase co-factor</td>
</tr>
<tr>
<td>M, Matrix</td>
<td>Form a layer underneath the cell membrane</td>
<td>Involve in viral assembly</td>
</tr>
<tr>
<td>SH, Small hydrophobic</td>
<td>Membrane</td>
<td>Form cation-selective channels and permeabilize membranes</td>
</tr>
<tr>
<td>G, Glycoprotein</td>
<td>Membrane</td>
<td>Attachment to host cells</td>
</tr>
<tr>
<td>F, Fusion protein</td>
<td>Membrane</td>
<td>Fusion of virion and host membrane, viral entry and syncytia formation</td>
</tr>
<tr>
<td>M2-1</td>
<td>Nucleocapsid and polymerase complex</td>
<td>Transcription anti-termination factor</td>
</tr>
<tr>
<td>M2-2</td>
<td>Nucleocapsid and polymerase complex</td>
<td>Transcription faction, RNA regulation</td>
</tr>
<tr>
<td>L, Large protein</td>
<td>RNA polymerase complex</td>
<td>Enzymatic activity of the polymerase</td>
</tr>
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Table 1.2: Viral proteins of RSV and their functions.

1.2.5 Respiratory syncytial virus life cycle

The life cycle of RSV is shown in Figure 1.7. The infection of ciliated respiratory epithelial cells by RSV is initiated by the binding of G protein to CX3CR1 on the apical cell surface. Then RSV F binds to nucleolin at the cell surface and fuses RSV membrane with the host cell membrane. The cellular entry is triggered. Another way for virus entry
is through actin-dependent macropinocytosis. After cellular entry, the nucleocapsid of the virus is released into the cytoplasm, where the virus transcription and replication begin. A replication complex composed by L-polymerase, P and M2-1 proteins, and the essential N-RNA nucleocapsid is formed. 10 capped, methylated and polyadenylated mRNAs are transcribed by using the viral RNA as template, and then are translated into 11 viral proteins by using host cell ribosome complex. The RSV RNA-dependent RNA polymerase not only transcribes mRNA for protein translation, but also can synthesize a full-length positive sense antigenome as the intermediate for replication. M2-2 is believed to be involved in the process. The complete negative-sense genomes are replicated using this antigenome as template. The surface glycoproteins are transported through the secretory pathway to the apical surface of host cells, while the other proteins remain in the cytoplasm. M protein binds to G and/or F, and also to the nucleocapsid. Viral proteins will assemble into filaments at the cell surface, containing both viral proteins and genomic RNA. After assembly, the virus buds from the lipid rafts on the apical membrane (55).
Attachment to the host cell membrane by G protein initiates the viral lifecycle. Virus entry into host cell via fusion by F protein or triggers the endocytosis by macropinocytosis. After enter into the host cell, viral genome is released into the cytoplasm. Transcription and translation happen, as well as replication. Finally, viral proteins and viral genomic RNP complex traffic to the apical cell membrane for assembly and budding, and release the newly formed virus.

1.2.6 Respiratory syncytial virus and immune responses

The replication of RSV can be inhibited by cell apoptosis, innate immune responses, and cell stress responses. The cell apoptosis includes both the extrinsic pathway and intrinsic pathway, triggering the activation of caspase3 and finally cell death. The extrinsic pathway is mediated by the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its death receptor 4/5 (DR4/DR5). The intrinsic pathway is
mediated by the B-cell lymphoma 2 (Bcl-2) protein. RSV proteins NS1, NS2, P, and SH can function in avoiding or delaying the cell death caused by apoptosis. Innate immune response is the first line to defense against and clear the virus. Several intrinsic pattern recognition receptors (PRRs) involve in this response, including Toll like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) (56). These PRRs recognize the virus and activate their downstream transcription factors, such as nuclear factor (NF)-κB and interferon regulatory factors (IRFs). NF-κB and IRFs can regulate the expression of pro-inflammatory cytokines and type I/III IFNs. The type I/III IFNs then activate the JAK/STAT-mediated signaling pathway, which will increase the expression of interferon-stimulated genes (ISGs). ISGs can repress the virus replication, and ultimately promote virus clearance. Virus has evolved some mechanisms to escape the innate immune response. For example, NS1 and NS2 will suppress the expression synthesis and signaling of type I/III IFNs. There are three kinds of cell stress, including ER stress, transcriptional stress, and oxidative stress. ER stress is caused by the overloading of glycoproteins F, G, and SH in ER/Golgi pathway; and can restrict virus replication by activating the protein unfolding mechanism. Transcriptional stress involves the formation of stress granule (SG), a complex of stalled mRNAs with proteins. SG can prevent the virus translation. Reactive oxygen species (ROS) is produced in oxidative stress, and can activate NF-κB and IRF3 (57).

Except the innate immune responses, adaptive immune responses are also important defenses against RSV infection, including neutralizing antibodies produced by the humoral immune response and specific cell-mediated immune response (lymphocyte transformation and cytotoxic T-cell responses). Neutralizing antibodies elicited by the F
and G proteins can not only help to resolve the virus, but also prevent reinfection by recognizing and binding to the viral epitopes required for virus entry and infection. Both CD4+ and CD8+ T cells are important for the RSV clearance from the respiratory tract. CD4+ T cells play an important role in helping the generation of high-affinity antibodies by B cells. CD8+ T cells can recognize and kill RSV-infected cells. It is of great importance in viral clearance (58).

1.3 Respiratory syncytial virus fusion glycoprotein (RSV F)

1.3.1 The Role of RSV F glycoprotein

   The fusion protein F is one of the transmembrane glycoproteins of RSV. It is responsible for the fusion of viral envelope with the host cell plasma membrane. This process is important for the entry of virus into the host cells, releasing the virus nucleocapsid into the cytoplasm of the host cells. F protein also promotes the fusion of an infected cell with its neighboring non-infected cells, leading to the formation of syncytium, a multinucleated giant cell (59). Other viral proteins are not necessary for the function of F. However, G protein and SH protein may enhance the fusion mediated by F and also the formation of syncytia (60,61).

1.3.2 RSV F glycoprotein conformations and structural information

   The F protein is a type I transmembrane glycoprotein with 5 or 6 N-linked glycans. Similar as other F proteins of Paramyxovirinae, RSV F is synthesized as an
inactive F0 precursor that is activated by a furin-like protease in the host cells. Three F0 monomers assemble into a trimer. But unlike other Paramyxovirinae F proteins that have only one furin cleavage site, there are two furin cleavage sites in RSV F, one at residue 109 (RARR \_ E, site I) and the other one at residue 136 (KKRKRR \_ F, site II). The site at residue 136 corresponds to that found in other Paramyxovirinae F proteins. The furin cleaving processes happening at both sites are independent (62). This two-stage cleavage will generate two subunits F2 and F1, and a 27-amino acid peptide in between. It has been pointed out the cleavage at site II is required for the conformational change of RSV F (63).

The intervening 27 amino acid peptide, called pep27, will be released after fully cleavage. The F2 subunit is proved to be the determinant of RSV host cell specificity (64). The F2 and the F1 subunits are covalently linked together by two disulfide bonds. Then the protein is functional active by exposing the Fusion Peptide, a hydrophobic peptide locating at the N-terminal of F1 subunit. Fusion peptide is inserted into the target membrane during fusion. Except this fusion peptide, F protein also has another two hydrophobic regions. One locates at the N terminal domain, and functions as the signal peptide for translocation into the ER. The other one is the transmembrane domain, near the C terminus (63).
Figure 1.8: Diagrammatic representation of the F protein.
F protein is composed of a cytoplasmic tail (CT), the transmembrane domain (TMD), the two heptad-repeat regions (HRA and HRB), a fusion peptide (FP), and the signal peptide (SP). The furin cleavage sites (at aa109 and 137) and positions of N-glycans (indicated with grey arrows) are shown.

The RSV F is expressed on the surface in a metastable prefusion form. It will be triggered to undergo conformational change to a more stable postfusion form. The conformational change of RSV F is important for facilitating the merge of viral and host membranes.

Prefusion and postfusion conformation

The crystal structures of RSV F in both prefusion and postfusion forms have been solved. All the structures were solved using the soluble F protein without its transmembrane domain and cytoplasmic domain.

In 2011, two independent groups simultaneously solved the crystal structure of the postfusion RSV F protein (65,66). The first 9 or 10 amino acids of fusion peptide were deleted to avoid the aggregation and rosette formation. The structure is similar to the previously solved human parainfluenza virus 3 (hPIV3) postfusion F structure (67). It is a cone shape structure, with a globular head and an extended stalk. This C3 symmetric
structure is composed of three F2/F1 protomers. The globular head is composed by \( \alpha \)-helices and \( \beta \)-sheets of the F2 and F1 subunits. The stalk region has a six-helix bundle, which is the key characteristic of the postfusion state. The six-helix bundle is composed of 3 HRA in the center and 3 HRB outside. HRB helices pack against HRA helices and bury them in the center. In this stable six-helix bundle, the N-terminal and C-terminal domain of F1 subunit are brought to close each other. That means in the full-length F protein, the hydrophobic fusion peptide (resides in N terminal of F1) inserted into the target cell membrane and the transmembrane region (C terminal of F1) linked with virus membrane are in close contacts for membrane fusion.

Later in 2013, McLellan et al. solved the prefusion form of RSV F by adding a C-terminal fibritin T4 trimerization motif to the soluble RSV F (aa 1-513) and complexing with a prefusion F specific neutralizing antibody named D25 (68). The prefusion F is a lollipop-shaped structure. Each monomer in prefusion F is divided into two lobes. Each lobe contains both the F2 and F1 subunits. The F2 N-terminal, which is mostly \( \beta \)-strand, is in the membrane-proximal lobe. Its C-terminal is mostly \( \alpha \)-helices, and is extends into the membrane-distal lobe. The N-terminal of F1 containing the fusion peptide is buried in the center of the trimer cavity.

By comparing the prefusion structure with the postfusion structure, we can see a dramatic conformational change. The fusion peptide and the five secondary-structure elements (\( \alpha 2, \alpha 3, \alpha 4, \) and \( \beta 3/\beta 4 \) hairpin) connected to it are rearranged and refolded into an extended single \( \alpha \)-helix in the postfusion state. This rearrangement exposes the hydrophobic fusion peptide from the interior to facilitate its insertion into the target cell membrane. The C-terminal of F1 also undergoes a reorganization. It moves towards the
N-terminal and brings the HRB closely packing HRA to form the six-helix bundle in the postfusion state.

Figure 1.9: Crystal structure of RSV F in prefusion conformation.
Left: Ribbon representative of RSV F prefusion trimer structure. Right: Ribbon representative of RSV F prefusion protomer. Some select secondary structure elements (α2, α3, α4, α5, α10, and β3/β4 hairpin) are labeled. (PDB: 4JHW)
A key characteristic of conformational change from prefusion form to postfusion form is that the two heptad repeats (HRA and HRB) will refold to close each other and form a six-helix bundle. The free energy released during this change is required for membrane fusion. Another heptad repeat HRC, an amphipathic α-helix in F2 subunit, which locates at the interface between two protomers of the prefusion trimer, also plays an important role in membrane fusion. It may stabilize the globular head of the prefusion F trimer, and involve in triggering the fusion (69).
Cytoplasmic domain of RSV F

RSV F has a 26 amino acids cytoplasmic tail (CT) at its C terminal domain, just adjacent to the transmembrane domain (TMD). Like fusion proteins of other paramyxoviruses, there is a palmitoylation site at cysteine residue 550 (C550) of the cytoplasmic tail (70). It is the only one cysteine in the cytoplasmic tail and is the site of addition for a palmitoyl group. Palmitoylation may be needed in virus assembly by affecting the trafficking to lipid rafts. In addition, the cytoplasmic tail affects the distribution pattern of F in the plasma membrane. When the cytoplasmic tail was modified, F protein evenly distributed rather than predominantly localized on filaments. The cytoplasmic tail is essential for the production of infectious virus (71). But unlike other fusion proteins, palmitoylation is not required for RSV F protein fusion. C550 mutation or the entire cytoplasmic domain deletion didn’t affect the protein processing and fusion. That means the cytoplasmic domain of RSV F is not essential for cell fusion (72).

Glycosylation sites of RSV F

RSV F has 5 to 6 N-linked glycosylation sites. The glycans of RSV F is N-linked glycans, different from those of G protein, which are mostly O-linked glycans. The F2 subunit has two N-linked glycan sites at Asn27 and Asn70. The F1 subunit has only one N-linked glycan site at Asn500. This glycosylation site is in HRB, adjacent to the transmembrane domain. Pep27 is predicted to have 2 or 3 N-glycosylation sites (Asn116, Asn120, and Asn126) depends on different RSV subtypes. The two glycans of F2 subunit
have minor impacts on the fusion activity of RSV F. But the only one glycan of F1 subunit is essential for the protein to cause membrane fusion by interacting with HRB (73).

The N-linked glycans are firstly formed in the ER, with a mannose core attaching mannose residues. These glycan residues are replaced by other more complex terminal glycans, such as N-acetyl-glucosamine and fucose, in the Golgi apparatus. This process is called as glycan maturation. The maturation of the glycans is not required for virus assembly, but is required for the cell fusion induced by F protein, and thus is important for virus infectivity (74).

1.4 Advances in RSV antiviral drug and vaccine development

RSV is a highly infective human pathogen, but currently there is no RSV vaccine approved and the options to treat RSV infected diseases are limited. There are many challenges in the development of high-quality and effective RSV vaccines. Firstly, the natural protective immunity against RSV is short lived, and reinfection is likely to happen. It is difficult to develop a long-lasting effective vaccine. Secondly, the immune response caused by RSV infection is not completely understood. It is relatively difficult to decide the potential population and also difficult to determine the administration frequency of a vaccine. Thirdly, the lack of good animal models of RSV infection makes it difficult to investigate the immune response for a specific vaccine. Last but not the least, the genome of RSV is susceptible to mutations, as the RNA polymerase of RSV cannot proofread or edit. But practically, most vaccine researches are still using the prototype virus strain.
Small molecule is an option to treat RSV infection. There is only one approved small molecule against RSV, Ribavirin, a panantiviral drug. The mechanism of action of Ribavirin is unclear. Because of the potential of toxic effects, limited efficacy, and high cost, the usage of ribavirin is restricted (75).

Using RSV-specific neutralizing antibodies as immunoprophylaxis is another effective approach to prevent RSV infection. There is only one FDA approved RSV-specific antibody for protection of a subset of premature infants, named as Palivizumab, which will be discussed later.

1.4.1 RSV F glycoprotein antigenic sites and related antibodies

The surface glycopolypeptides F and G are the major targets of immune responses caused by RSV infection. They are the only two antigens to trigger adaptive immune responses to produce neutralizing antibodies and also the major components of a protective vaccine. G protein is poorly conserved, with only 55% identity of amino acid sequence between RSV/A and RSV/B. Thus, the neutralizing antibodies targeting G protein are not effective enough. On the contrary, F protein is highly conserved with approximately 90% identity and is more stable. Undoubtedly, F protein is a better antigen in immunity research and vaccine development.

There are more than 35 mAbs targeting RSV F reported, representing 22 unique functional epitopes. Some antibody epitopes remain accessible and unchanged in both pre- and post-fusion F. The majority of epitopes with functional antibodies reported are in the prefusion form or accessible in both pre- and post- forms (76).

Even if there is dramatic structural difference between the prefusion and postfusion forms of RSV F, the majority of the secondary and tertiary structures are
conserved. Various antigenic sites have been described for the pre- and post- fusion RSV F and distinguished as sites ø, I, II, III, IV, and V (Figure 1.11).

Figure 1.11: Antigenic sites of RSV F prefusion and postfusion structures.
Left panel: surface representation of RSV F trimer with antigenic sites colored.
Right panel: Ribbon representation of RSV F protomer with antigenic sites colored. Antigenic sites are highlighted in different colors: site Ø, red; site I, blue; site II, yellow; site III, blue; site IV, magenta; and site V, orange. Site Ø is unique in prefusion RSV F.
**Antigenic Site II**

Antigenic site II is corresponding to amino acid 255 – 275, as a helix-loop-helix structure. It is conserved in both pre- and post-fusion F. Both Palivizumab and Motavizumab are RSV F site II specifically antibodies.

Palivizumab (Synagis®) is a humanized murine mAb derived from mAb 1129 and is the only licensed anti-infective monoclonal antibody for RSV infection (77). It is approved for prevention of severe lower respiratory tract disease caused by RSV in a subset of premature infants (78). Using palivizumab as immunoprophylaxis for RSV infected disease is effective but costly. Motavizumab (Numax) is a second-generation humanized mAb derived from palivizumab. It has 13 amino acids different from palivizumab. It is approximately 10-fold more potent than palivizumab. But motavizumab was refused by the FDA for marketing, because it didn’t work better than palivizumab and has more side effects (79). Crystal structure of motavizumab in complex with a 24-residue peptide corresponding to amino acid 254-277 of RSV F was solved (80). Motavizumab has higher shape complementarity and electrostatic potential with this peptide. Motavizumab heavy chain forms hydrogen bonds with RSV F as F/Asn262-Fab/Asp54, F/Asn262-Fab/Lys56, F/Ser275-Fab/Ile97. There is also a hydrogen bond formed between the light chain and RSV F, as F/Asn268-Fab/Gly90. A salt bridge is formed between the side chain of F/Lys272 and that of Fab/Asp49. Figure 1.12 shows the superimposed model of motavizumab with RSV F in post-fusion form and the detailed interactions.
Figure 1.12: Structure of Motavizumab Fab bound to postfusion RSV F protein.
(A) Ribbon representation of the overall structure of Motavizumab bound with RSV F postfusion by superimposing 3IXT to 3RRR. RSV F protein is colored as red, yellow and green for each protomer. Motavizumab is colored as light orange for light chain (LC) and light green for heavy chain (HC).
(B) Detailed interactions between Motavizumab and RSV F protein site II motif (PDB: 3IXT). Several key hydrogen bonds important for molecular recognition are drawn as dark dashed lines, with residues labeled.

Mousa et al. further designate antigenic site II to IIa, IIb, and VII. Antibodies targeting site IIa and IIb are neutralizing antibodies, while site VII is targeted by nonneutralizing antibodies. The difference between site IIa antibodies and site IIb antibodies is that the latter can compete with both neutralizing and nonneutralizing antibodies. According to this classification, motavizumab targets antigenic site IIa, while palivizumab targets antigenic site IIb.

14N4 is another neutralizing antibody specifically recognizes antigenic site IIb. Crystal structure of 14N4 Fab in complex with postfusion RSV F was solved to 4.1Å as shown in Figure 1.13 (PDB: 5J3D). Three 14N4 Fab molecules bind to the RSV F trimer, one at each protomer. The heavy chain of 14N4 Fab lies between the two helices of site II

Figure 1.13: Structure of 14N4 Fab bound to postfusion RSV F protein. (A) Ribbon representation of the overall structure of 14N4 in complex with RSV F (PDB: 5J3D). RSV F protein is colored as red, yellow and green for each protomer. 14N4 Fab is colored as pink for light chain and light blue for heavy chain. (B) Detailed interactions between 14N4 Fab and RSV F protein site II. Several key hydrogen bonds important for molecular recognition are drawn as dark dashed lines, with residues labeled.
**Antigenic Site IV**

Antigenic site IV is corresponding to amino acid 422 – 438, an uninterrupted fragment of the primary F sequence. But the location of site IV is different in pre- and post-fusion F. It locates relatively close to the membrane-proximal part in prefusion F, but is in membrane-distal part in postfusion F.

mAb 101F is a site IV antibody. Crystal structure of 101F Fab in complex with this epitope peptide is solved (82). Superimposing this structure with RSV F trimer suggests 101F may contact additional surfaces besides this linear peptide (Figure 1.14). The shape complementarity between 101F Fab and site IV peptide is high, but the electrostatic potential complementarity is weak. The side chain of RSV F Arg429 forms hydrogen bond with the Tyr96 of heavy chain of 101F Fab. There are hydrophobic contacts between RSV F Ile431/Ile432 and Phe98 of 101F heavy chain, besides the hydrogen bond between F/Ile431-101F/Phe98. The additional hydrogen bonds include F/Thr434-101F/Ile91, F/Thr434-101F/Ile92, F/Phe435-101F/Asp94, F/Ser436-101F/Arg56, F/Ser436-101F/Asp94.
Figure 1.14: Structure of 101F Fab bound to postfusion RSV F protein.
(A) Ribbon representation of the overall structure of 101F Fab bound with RSV F postfusion by superimposing 3O45 to 3RRR. RSV F protein is colored as red, yellow and green for each protomer. 101F Fab is colored as green cyan for light chain and deep salmon for heavy chain.
(B) Detailed interactions between 101F Fab and RSV F protein site IV peptide (PDB: 3O45). Several key hydrogen bonds important for molecular recognition are drawn as dark dashed lines, with residues labeled.

Both antigenic site II and IV are present on the pre- and post-fusion conformations. Thus, it is thought the neutralizing activity of antibodies target these two sites is likely as a result of steric hindrance of the membrane fusion, not of the F refolding prevention.
Antigenic site ø

Antigenic site ø exclusively exists in prefusion F state. It locates at the membrane-distal apex of prefusion F. When RSV F transformed from prefusion to postfusion, the secondary structure of antigenic site ø remains unchanged, but the tertiary structure changes dramatically. Antibodies D25, AM22, and 5C4 specifically bind to this unique antigenic site. McLellan JS et al. solved the crystal structure of D25 in complex with the prefusion RSV F (68). D25 binding can stabilize RSV F in its prefusion conformation. The heavy chain of D25 interacts with one protomer with its light chain binding to the same protomer as well as the neighboring protomer. The neutralizing activity of antibodies preferentially target antigenic site ø probably results from stabilizing the RSV F in prefusion state and preventing the conformational change.

Figure 1.15: Ribbon representation of crystal structure of D25 in complex with prefusion RSV F.
The D25 Fab is colored as violet for heavy chain and teal for light chain. The prefusion RSV F is colored as yellow, green and red for each protomer. (PDB: 4JHW)
Antigenic Site VIII

There is another antigenic site, designated as antigenic site VIII. This site corresponds to residue 163-181 of RSV F, and lies between antigenic site II and antigenic site ø in the prefusion form. hRSV90 is a site VIII antibody, it can compete with both site II and site ø antibodies. The structure of hRSV90 in complex with prefusion RSV F has been solved (83). Each hRSV90 Fab molecule interacts with one protomer of RSV F. Both heavy chain and light chain of hRSV90 have specific interactions with ‘helix-loop-sheet’ motif of site VIII. In addition, the heavy chain also interacts with site ø, while the light chain has additional interactions with site II. This helix-loop-sheet structure will rearrange and become part of the extended helix of HRA in postfusion conformation. That’s why hRSV90 only specifically recognizes prefusion RSV F.

Figure 1.16: Ribbon representation of crystal structure of hRSV90 in complex with prefusion RSV F.
The hRSV90 Fab is colored as cyan for heavy chain and orange for light chain. The prefusion RSV F is colored as yellow, green and red for each protomer. (PDB: 5TPN)
Prefusion RSV F Quaternary Epitope

Another group of antibodies recognize a quaternary epitope specifically exists in prefusion RSV F. AM14 is the example for this group of antibodies. AM14, a human antibody, recognizes a quaternary epitope spanning two prefusion RSV F protomers. The crystal structure of AM14 in complex with prefusion RSV F is solved by Gilman et al. to 5.5 Å in the presence of motavizumab (84). Three AM14 Fab molecules and three motavizumab molecules bind to prefusion F trimer, but at different antigenic sites.

Figure 1.16 shows the structure of three AM14 Fab molecule bound to the RSV F trimer in prefusion. AM14 binds exclusively to the junction surface formed by two RSV F protomers. In addition, AM14 specifically recognizes the cleaved prefusion RSV F trimer. The existence of Pep27 may hinder the binding of AM14 to RSV F. And the uncleaved RSV F may adopt a native trimeric state that AM14 couldn’t recognize.

Figure 1.17: Ribbon representation of crystal structure of AM14 in complex with RSV F trimer in prefusion form.
The AM14 Fab is colored as pink for heavy chain and blue for light chain. The prefusion RSV F is colored as yellow, green and red for each protomer. (PDB: 4YZP)
The structural information on these antigenic sites and the discovery of novel neutralizing antibodies can shed a light on the novel antibody and vaccine development and promote the immunoprophylaxis for RSV F infected diseases.

1.4.2 Small molecules with antiviral activity against RSV F

Using small molecule to inhibit important proteins in RSV infection process is another therapeutic option for RSV infected disease. There are several therapeutic small molecules been developed to bind RSV F and inhibit the fusion process. JNJ-2408068, TMC-353121, and BMS-433771 are prefusion RSV F specific inhibitors. The structures of these inhibitors bound to a prefusion RSV F reveal that these inhibitors bind to a pocket inside the prefusion RSV F cavity to stabilize the metastable conformation. There are two binding modes, one is binding to two of the three lobes within the binding pocket, as JNJ-2408068 and BMS-433771; the other one is binding to all three lobes, as TMC-353121. The key interactions are formed between the planar aromatic groups of the inhibitors and the aromatic side chains of Phe140 in RSV F fusion peptide and Phe488 in the HRB region. In addition, a long positively charged group of JNJ-2408068 and TMC-353121 interacts with a negatively charged pocket formed by Asp486 and Glu487 in RSV F (85). These small molecules can be seen as antagonists for membrane fusion by hindering the rearrangement of fusion peptide and HRB. The structural information on small molecular inhibitor binding with RSV F is important in identifying potential drug binding sites in the F protein.
Chapter 2

Near-atomic structure of a novel site II/IV antigenic epitope on respiratory syncytial virus fusion glycoprotein

2.1 Introduction

Human respiratory syncytial virus (hRSV) is a leading cause of pneumonia and bronchiolitis in premature newborn, young children, and adults over 65 years old. Globally, RSV is responsible for over 30 million lower respiratory tract infections (LRTI), 3 million hospitalizations, and 50-75,000 deaths each year in children under 5 years. Newborns under age 6 months account for over a 1 million hospitalization and 27,300 deaths with the majority of infant deaths in developing countries (4,5). In the United States, RSV is responsible for 2 million outpatient visits and nearly 60,000 hospitalizations in children under 5 years of age (86). In adults over 65 years, there are 177,000 hospitalizations and 14,000 deaths each year in the United States (87,88).

There is no approved RSV vaccine. Palivizumab (Synagis®) is currently the only immuno-prophylaxis approved for prevention of RSV infection in premature newborns. The high cost, 5-dose treatment regimen, and limited efficacy prevents its use and availability in developing countries (77,89,90). There is clearly a great need for a preventative vaccine or a new antibody.

The G and F glycoproteins are primary targets of the immune response. G protein is heavily O-glycosylated with over 60% of its 90 kD mass comprised of carbohydrate. G
glycoproteins are heterogeneous with limited sequence homology (53%) and little antigenic cross-reactivity between RSV/A and B subgroups (8,91-93). In contrast, F glycoprotein sequences are conserved (>90%) with a high degree of antigenic cross-reactivity between RSV/A and B subgroups (94). Thus F glycoprotein is a preferred target for vaccine development.

F glycoprotein is a 574 aa polypeptide (F0) with a molecular weight of 70 kD. F0 is post translationally processed by a host cell furin-like protease that cleaves at residues 109 and 136, producing three peptide-fragments. The intervening 27 aa fragment (p27) is removed exposing the hydrophobic fusion peptide (FP). The amino-terminal F2 fragment (17 kD) and the larger carboxy-terminal F1 fragment (48 kD) comprise the surface exposed ectodomain. F1 and F2 are covalently coupled through two disulfide bonds at positions C70-C212 and C37-C439. The F2 fragment has two glycans at residues N27 and N70 and the F1 subunit has a single glycan at residue N500. F monomers assemble to form trimers which are anchored in the virus envelope via a hydrophobic transmembrane domain (TM) located at the carboxy-terminus of the F1 fragment.

The F glycoprotein is present in two distinct conformations on the virus surface. The pre-fusion (pre-F) conformation is unstable and undergoes unidirectional rearrangement to a stable post-fusion (post-F) conformation. The rearrangement exposes the FP which is essential to fuse of the virion to the host cell endosomal membrane and transit of the viral genome into the infected cell (51).

Antibodies that prevent RSV fusion were first identified over 30 years ago (95). The realization that neutralizing antibodies afford protection against RSV infection lead to development of the humanized monoclonal antibody (mAb) palivizumab, which is
currently the only approved immune-prophylaxis for treating premature newborns at risk of severe LRTI (96). Since then, the crystal structure of the F glycoprotein pre-F and post-F conformations in complex with murine and human mAbs has resulted in the identification and fine mapping of a number of antigenic epitopes. Antigenic site II is the target for neutralizing antibodies palivizumab and hRSV 14N4 (site IIa) and the optimized antibody motavizumab and hRSV 3I20 (site IIb) (81). Antigenic site II is located on the F1 fragment spanning amino acids (aa) 257-277. In addition, non-neutralizing site II binding human mAbs (4B6, 9J5 and 12I1) have been reported and found to bind to bind site II at an angle different from palivizumab and motavizumab. This epitope has been designed antigenic site VII (81). Antigenic site IV has been mapped to F1 and spans aa 422-438 on the globular head region. Site IV neutralizing mAbs include 101F (65,82,97), humanized RSHZ19 (98) and murine R1.42 (99). Sites II, IV and VII epitopes are conserved and are present on both the pre-F and post-F conformations (66,97). Antigenic site zero (Ø) is found only on the RSV F pre-F conformation. This antigenic site is bound by prototype human mAbs D25 and AM22 (100) and murine 5C4 (68), which are highly neutralizing. Site Ø epitope is conserved within the RSV/A subgroup and less conserved within the RSV/B subgroup (101). This epitope is a conformation-dependent quaternary epitope consisting of F2 aa 62-69 and F1 aa 196-210 that are juxtaposed on the pre-F conformation. Transition of the pre-F to post-F conformation brings the F1 and F2 binding sites out of position thus disrupting the site Ø epitope (68). Site VIII was recently identified with human hRSV90. Crystal structure reveals the hRSV90 heavy chain binds to site Ø and the light chain to amino acids near site II (83).
In this chapter, we characterized a series of monoclonal antibodies. Epitope binning studies demonstrate that one antibody named R4.C6 competes the binding of antibodies to antigenic sites IIa/b, VII and IV. Single particle cryo-EM and 3D reconstruction was use to define the binding sites of R4.C6 Fab in complex with RSV F. The near-atomic structure indicates this murine mAb R4.C6 binds to a novel neutralizing epitope involving both antigenic sites II and IV. R4.C6 heavy chain CDRs and light chain CDRs interact directly with the antigenic site II on one protomer and the site IV located on a neighboring protomer of the F glycoprotein trimer. (Note 1)

2.2 Materials and Methods

2.2.1 Cell lines, viruses, RSV F nanoparticles, antibody reagents, and synthetic peptides

HEp-2 cells (ATCC CCL-23) were maintained in MEM with Earle’s salts, L-glutamine (Gibco Laboratories, Gaithersburg, MD, USA), 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and antibiotics (Life Technologies, Grand Island, NY, USA). *Spodoptera frugiperta* Sf9 cells (ATCC CRL-1711) were maintained in Grace’s Insect Medium containing yeast extract, a lipid mixture with 10% FBS and antibiotics. *Trichoplusia ni* High Five cells (BTI-TN-5B1-4, ATCC CRL-10859) were maintained in Insect-XPRESS Medium with L-glutamine and antibiotics (Lonza, Walkersville, MD USA).
RSV/A Long (ATCC, VR-26) reference strain was obtained from ATCC (Manassas, VA, USA). Virus stock was prepared from clarified supernatants and stored at -80°C in PBS with 25% sucrose as a cryo-protectant.

RSV F nanoparticles (NP) were produced in Sf9/baculovirus expression system by a previously described method (102,103).

Palivizumab (Synagis®, MedImmune, Inc., Gaithersburg, MD, USA) and Motavizumab (National Institute of Standards and Technology, Gaithersburg, MD, USA) were obtained commercially or from NIST. D25 (Creative Biolabs, Shirley, NY, USA) and RSHZ19 (Absolute Antibody, Oxford, UK) (104) were purchased commercially. hRSV14N4, hRSV3J20 and hRSV90 (81) were provided by Dr. J. Crowe, Vanderbilt University (Nashville, TN, USA). Palivizumab site II synthetic peptides were commercially synthesized by Peptide 2.0 (Chantilly, VA, USA).

### 2.2.2 Generation of monoclonal antibodies

RSV F-specific mAbs were generated using standard methods (105). Briefly, female BALB/c mice were immunized by interperitoneal injection with 5 µg RSV F nanoparticles. Four days after boosting, spleens were collected and single cell suspensions of splenocytes pooled and depleted of IgM B lymphocytes with a magnetic cell sorting system (Miltenyi Biotec, Auburn, CA, USA). IgG enriched splenocytes were fused with P3X63.Ag.6.5.3 myeloma cells (105). Hybridomas were screened with an RSV F ELISA and positive cultured cloned by limiting dilution. Hybridoma cell lines were expanded in 75 cm² T-flasks in serum free media.
2.2.3 Monoclonal antibody purification and Fab preparation

Monoclonal antibodies were routinely purified from spent medium with a protein G affinity column using manufacture’s protocol (GE Healthcare Life Sciences). To produce R4.C6 Fab, the hybridoma was grown in serum free medium in a 7 L bioreactor by Southern Biotech (Birmingham, AL, USA). The spent supernatant was concentrated and applied to a protein-G affinity column. Purified R4.C6 antibody was digested with papain (Pierce Fab Preparation Kit, Thermo Fisher Scientific) with mild reduction. Antigen-binding (Fab) fragments were purified from undigested IgG and Fc fragments by ion exchange chromatography (Southern Biotech, Birmingham, AL, USA). The purified Fab was 98.8% as determined by reverse phase HPLC.

2.2.4 RSV F710 construct and its mutants

RSV F710 construct

The RSV F710 construct contains amino acids 26-526 of the fusion (F) glycoprotein ectodomain derived from the RSV A2 F gene sequence (Genbank Accession No. U63644) linked by a baculovirus gp64 signal peptide (SP). Ten amino acids were deleted (ΔPhe137-Val146) within the fusion peptide domain (FD) and the second furin cleavage site at aa131-136 was mutated as KKQKQQ to KKRKRR (102). The transmembrane domain (TM) was deleted and replaced with the T4 fibritin foldon trimerisation motif (GSGYIPEAPRDQAYVRKDGEWVLLSTFL). The synthesis RSV A2 F gene was codon optimized for expression in Sf9 insect cells. This gene fragment was cloned into the BamHI/HindIII sites of pFasbacDUAL vector (Thermo Fisher, Waltham, MA, USA) downstream of the AcMNPV polyhedron promoter, with a 6-
histidine tag (6-His) at the C terminal separated by a thrombin cleavage site. The Sf9 furin sequence fragment (Genbank Accession No. CAA93116.1) was cloned between Xmal/KpnI sites of the same pFastbacDUAL vector downstream of AcMNPV p10 promoter. The RSV F710 protein expressed by using this construct is schematically shown in **Figure 2.1**.

**Figure 2.1: Schematic shown the expressed RSV F710.**
The RSV F710 consisting of amino acid 26-526 from the RSV A2 F ectodomain linked by a baculovirus gp64 signal peptide (SP) that will be removed after expression is modified by removal of the C terminal transmembrane (TM) domain and replaced with the T4 fibritin foldon trimerisation motif (FF) and a 6-histidine tag (6H) to facilitate purification. The second furin cleavage site is mutated as KKQKQQ to KKRKRR, and 10 amino acids was deleted in the F1 N-terminal fusion peptide domain (FD).

**RSV F710 Mutants**
Constructs harboring the mutations of T267A/N268G/D269G/K272A at antigenic site II (Mutant/SiteII), the mutations of N428G/N454G at antigenic site IV (Mutant/SiteIV), and the mutations at both antigenic sites (Mutant/SiteII&IV), respectively, were made by using QuickChange Multi Site-directed mutagenesis method. All the constructs were confirmed by sequencing.
2.2.5 RSV F710 protein purification

The recombinant baculoviruses were made according to the Bac-to-Bac baculovirus expression system instruction (Invitrogen, Carlsbad, CA, USA). Briefly, baculovirus bacmid DNA was generated by site-specific homologous recombination following transformation of pFastBacDUAL-based transfer plasmid containing RSV F710 and furin gene into E. coli DH10Bac competent cells (Thermo Fisher, Rochester, NY, USA), which contained the Autographa californica multinuclear polyhedrosis virus (AcMNPV) genome. Recombinant bacmid DNA was extracted from E. coli cells and transfected into Sf9 cells using Cellfectin™ II (Invitrogen, Carlsbad, CA, USA). The transfection supernatants containing the recombinant baculovirus (BV) were harvested and amplified. The titers of BV stocks were determined by using plaque assay.

Cultures of HighFive cells at $1.5 \times 10^6$ cells/ml cell were infected with the recombinant baculovirus RSV F710 at multiplicity of infection (MOI) = 2 pfu per cell then incubated at 27°C for 48-60 hours. Cell culture supernatant containing secreted soluble RSV F710 was collected by centrifugation for 30 min at 4000 × g. The supernatant was then dialyzed against 20 mM Tris, pH 7.0, 50 mM NaCl followed by incubating with Ni-NTA resin (Thermo Scientific, Rochester, NY, USA). The protein-bound Ni-NTA resin was washed with wash buffer 20 mM Tris, pH 7.0, 150 mM NaCl, 25 mM Imidazole and eluted with elution buffer 20 mM Tris, pH 7.0, 150 mM NaCl, 250 mM Imidazole. The elutions containing RSV F710 protein were concentrated and buffer exchanged to decrease the NaCl concentration to 100 mM. The protein was then purified by Mono S 5/50 GL (GE Healthcare, USA) with buffer 20 mM Hepes, pH 7.0 and NaCl
gradient from 50 mM to 1 M. Superdex 200 10/300 GL (GE Healthcare, USA) was used for further purification with buffer 20 mM Hepes, pH 7.0 and 150 mM NaCl.

### 2.2.6 SDS-PAGE, western blot and protein concentration determination

RSV F710 purity was determined analyzed using 12% Bis-Tris SDS-PAGE gels under reducing and non-reducing conditions. Gels were stained with Coomassie Blue staining buffer. Gels were western blotted by transfer with a Turboblot (Bio-Rad, USA). RSV F710 was detected with anti-His HRP-conjugated probe and imaged using the chemiluminescence method with ECL reagents (BioRad, USA).

Protein concentration was determined based on the absorbance at 280 nm. The extinction coefficient Abs 0.1% (=1 g/L) is 0.877 for RSV F710 and 0.878 for RSV F710 mutant/SiteIV, respectively. The OD280 is measured using spectrophotometer with UV lamp and quartz cuvette. The equation to calculate the protein concentration is as following:

\[
Protein\ Concentration = \frac{OD_{280}}{Extinction\ coefficient \times Pathlength \times Sample\ Dilution}
\]

### 2.2.7 RSV F IgG and anti-palivizumab site II ELISA

RSV F and palivizumab site II peptide specific mAb were evaluated with an enzyme linked immunosorbent assay (ELISA). 96-well MaxiSorp microtiter plates (Thermo Scientific, Rochester, NY, USA) were coated with 2 μg/mL of purified RSV F
protein or palivizumab site II peptide (RSV F aa 255-276) overnight at 5±3°C. Plates were washed with phosphate buffered saline containing 0.05% Tween (PBST, Quality Biologicals, Gaithersburg, MD, USA) and unreacted surface blocked with Blocking buffer (5% milk in PBS, Quality Biologicals) for one hour at room temperature. Plates were washed with PBST and 2-fold serial dilutions (1:10 to 1:1,024,000) of purified mAb was added to coated plates in duplicate. Plates were incubated for two-hours at room temperature, washed with PBST, and bound antibodies detected by the addition of horseradish peroxidase conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL, USA). After a 1 hr incubation, plates were washed with PBST and TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Sigma, St. Louis, MO, USA). The reaction was stopped by the addition of TMB Stop Buffer (Scytek Laboratories, Logan, UT, USA) and plates read at 450 nm in SpectraMax plus plate readers (Molecular Devices, Sunnyvale, CA, USA). Data was analyzed using SoftMax pro software. A 4-parameter fit curve was used to analyze the data and antibody titer was defined as the reciprocal antibody dilution that resulted in a half maximal effective concentration for binding (EC₅₀).

2.2.8 Virus neutralizing ELISA

Virus neutralizing activity was determined with a micro-neutralizing assay. Briefly, purified mAbs were serially diluted over the concentration range 0.004 – 10 µg/mL in 96-well tissue culture plates and mixed with 200-350 TCID50 of RSV/A Long for 2 hours at 37°C. Low passage HEp-2 cells (2.5 x 10⁴ cells) were added to the antibody/virus mixture at 37°C in 5% CO₂ incubator for 4-5 days. The plates were washed and fixed with formalin. Infectious virus was detected by adding optimally
diluted mouse anti-RSV M2-1 (clone RSV 5H5) monoclonal antibody (Novus Biologicals, LLC, Littleton, CO, USA). The plates were washed and horseradish peroxidase (HRP) rabbit anti-mouse IgG added followed by addition of TMB (3,3′,5,5′-tetramethylbenzidine) substrate. The half maximal inhibitory concentration (IC₅₀) was determined by a 4-parameter curve fitting using Prism software.

2.2.9 Quantitative octet assay

Antibody cross-competition binding assay

Antibody cross-competition was performed by biolayer interferometry using an Octet QK384 system (Pall Forte Bio, Fremont, CA, USA). Histidine tagged RSV F710 protein (10 µg/mL) was immobilized on anti-penta-histidine biosensor tips. Captured RSV F710 was exposed to mAbs in two sequential steps. Biosensor tips were exposed to the first mAb (20 µg/mL) for 300 s followed by dipping the tips in the second antibody (10 µg/mL) for an additional 300 s. Assays were performed at 30°C with continuous agitation at 1000 rpm. If the binding of the second mAb to RSV F protein is prevented or reduced by the existence of the first mAb, the second mAb will be considered as competing for the similar epitope with the first mAb. Conversely, if the first mAb does not interfere with the binding of the second mAb, the antibodies will be considered as binding to distinct epitopes. The percent inhibition of antibody binding by the competing mAbs was calculated with Octet data analysis HT10.0 software. The percent inhibition of binding by competing mAbs was calculated by the following equation:

\[
%\text{Inhibition} = (100 - \frac{\text{Analyte mAb binding in presence of competitor mAb}}{\text{Analyte mAb binding alone}})\%\]
**R4.C6 Fab binding affinity of RSV F710 and its mutants**

R4.C6 Fab binding affinity of RSV F710 and its mutants was determined by kinetic analysis using an Octet RED96 system (Pall Forte Bio, Fremont, CA, USA). Histidine tagged RSV F710 protein and its mutants (20 µg/mL) were immobilized on Ni-NTA biosensor tips. The biosensor tips with RSV F proteins were subjected to associating with R4.C6 Fab in kinetic buffer (PBS with 0.01% bovine serum albumin) for 20 minutes and dissociating in the kinetic buffer (without R4.C6 Fab) for additional 20 minutes. Different R4.C6 Fab concentrations (5 nM, 10 nM, and 20 nM) were used to obtain a full range of association and dissociation curves. Assays were performed at 30°C with continuous agitation at 1000 rpm. The association rate ($K_{on}$) and the dissociation rate ($K_{off}$) were determined by fitting the binding curves using a 1:1 binding model. The equilibrium dissociation constant $K_D$ was calculated from $K_{off}/K_{on}$.

**2.2.10 Surface plasmon resonance (SPR)**

R4.C6 Fab binding affinities to site II synthetic peptides were determined by using surface plasmon resonance carried out on Biacore™ T200 instrument (GE Healthcare, Baltimore, MD). Protein A (Fisher Thermo Scientific, Waltham, MA) was immobilized on CM5 chips through amine-coupling reaction. Protein A was diluted to 100 µg/mL in an acetate buffer (pH 4) and injected at a flow rate of 10 µL/min for 10 minutes. The targeted coupling level was 300 response units (RU). R4.C6 Fab was diluted to 2 µg/mL and injected over the protein A immobilized CM5 chip at approximately 10 µL/min for 45 seconds. Binding of various site II synthetic peptides
was determined by injecting different concentrations of each peptide (5, 10, 20, 40 nM) over the antibody immobilized chip at 40 µL/min for 180 seconds followed by a 700 seconds dissociation time. The sensorgrams were analyzed by BIAcore kinetics analysis using a 1:1 fitting model to determine the kon and koff rates. Chips were regenerated by injection of 100 mM HCl at 40 µl/min for 45 seconds. The apparent equilibrium constant (K_a) was calculated from ratio of K_off / K_on.

2.2.11 R4.C6 Fab and RSV F complex preparation

To prepare R4.C6 Fab in complex with RSV F710 protein, purified RSV F710 protein was mix with R4.C6 at molar ratio of 3:4. This ratio can ensure the RSV F protein is saturated by R4.C6. The mixture was kept on ice for 30 minutes and then purified on a Superdex200 10/300 GL gel filtration column (GE Healthcare, USA) with a running buffer of 10 mM Hepes, 100 mM NaCl, pH 7.0. Purities of samples from each peak were analyzed by using 12% reduced Bis-Tris SDS-PAGE gel.

2.2.12 Negative staining for R4.C6 – RSV F710 complex

Carbon coated Formvar 200 mesh Copper grids (Ted Pella Inc. Redding, CA, USA) were glow discharged for 40 seconds, followed by overlaid with 3 ul R4.C6-RSV F710 complex at 0.01 mg/ml for 3 minutes. The grids were washed in water twice and stained with 2% uranyl formate for 1 minute. After air dry, the grids were checked using JEM2100 electron microscope (JEOL, Peabody, MA, USA) operating at 200 kV and
40,000x magnification. Images were taken using a Gatan 4k × 4k CCD camera (Gatan Inc. USA).

2.2.13 Preparation of graphene-oxide-support-covered grids for cryo-EM

Graphene oxide dispersion (Sigma-Aldrich, USA; 2mg/ml in H2O) was diluted to 0.2mg/ml with mqH2O and spun at 300x g for 30s to remove large aggregates. Quantifoil R1.2/1.3 200 mesh holey copper grids (Quantifoil, Jena, Germany) were glow discharged for 75s. 3ul of the graphene oxide suspension was added to the carbon side of the grid and incubate for 2 min. After incubation, the graphene oxide solution was removed by brief blotting with Whatman No. 1 filter paper from the side, followed by wash with mqH2O and blotting. The coverage of graphene oxide on the grid was visualized using TEM in low magnification mode before use, as shown in Figure 2.2. The graphene-oxide-coated grids can be used for plunge-freezing without any further treatment.
Figure 2.2: Graphene oxide covered grids.
(A) Quantifoil grid hole is covered by the graphene oxide flake.
(B) Quantifoil grid hole without graphene oxide covered.
(C) Overall graphene oxide coverage observed in low magnification mode at high defocus (100 mm), the brighter holes are covered with graphene oxide.

2.2.14 Preparation of Cryo-EM specimen

Specimens were plunge-frozen using Leica EM GP automatic plunge freezer (Leica Microsystems, Vienna, Austria) with its environmental chamber at 22°C and relative humidity at 98%. A 3.5μl aliquot of the complex diluted to 0.05mg/ml was applied onto Copper 200 square mesh Quantifoil R1.2/1.3 holey carbon grids (Quantifoil,
coated with graphene oxide. After 15s adhering, the grid was automatically blotted for 4s from the specimen side with Whatman No. 1 filter paper and immediately plunged from the environmental chamber into liquid ethane. Then the grids were transferred and stored in liquid nitrogen before imaging.

2.2.15 Cryo-EM data collection

All grids were screened on a JEM3200FSC cryo-electron microscope (JEOL, Peabody, MA, USA) operating at 300kV, with energy slit of the in-column filter of 20 eV. Images were recorded using K2 Summit direct electron detector camera (Gatan) in super-resolution electron counting mode at 30,000x microscope magnification (corresponding to a calibrated physical pixel size of 1.2546 Å). The dose rate is 5 electrons/Å2/sec and 50 frames were acquired in a total exposure time of 10 sec. A total of 2734 DDD images were collected.

2.2.16 Cryo-EM image processing

The dose-fractionated super-resolution raw image stacks were binned 2 X 2 by Fourier cropping resulting in a pixel size of 1.2546 Å for further image processing. Each image stack was subjected to motion correction using MotionCor2 (106), the whole frames (all 50 frames) of each image stack were used. Gctf (107) was used to estimate the contrast transfer function parameters. 7,456 particles were boxed out manually using ‘e2boxer.py’ in EMAN2 (108) as a particle subset to calculate reference-free 2D class averages, which was then used as templates for automated particle picking of the entire
date set. 543,639 particles were picked finally. An initial map was generated with 3-fold symmetry imposition from 2-D reference-free averages using EMAN2. This initial reference map was masked so that only the RSV F part was kept. It was then low pass filtered to 60 Å resolution as a starting point. RELION2.0 package (109) was used for subsequent imaging processing. The initial runs of 2D and 3D classifications were used to remove false positive particles from the auto-picking. Only those good particles were selected for further analysis. Several rounds of iterative 3D classification and 3D auto-refinement were performed. 234,479 particles were used in the final refinement to achieve a 3.9 Å resolution density map. A soft mask in RELION post-processing was applied before computing the FSCs. The final resolution was estimated by 0.143 cutoff of FSC. The density map was sharpened by applying a B-factor of -250 Å² estimated by an automated procedure. Local resolution variations were estimated with ResMap (110) using two independent maps.

2.2.17 Model building and refinement of the R4.C6 Fab - RSV F complex

Because of the six-helix bundle characteristic of RSV F seen in the cryo-EM density map, the crystal structure of post-fusion RSV F (PDB code: 3RRR) was docked into the map using Chimera. Since there is no known structure for mAb R4.C6, a homology model was built using the known structures of heavy chain and light chain variable domains with similar sequences (PDB code: 1I3G). The amino acid sequence identity between heavy chain of R4.C6 and homology model is 72%, and the identity for light chain is 81%. The molecular dynamics flexible fitting (MDFF) (111) method was used to flexibly fit the homology model into density map. The model for the complex was
then optimized using Phenix real-space refinement (112). Next, the model was slightly adjusted manually using Coot (113) to optimize the local fit into the density. Finally, a new method proposed by our group named as Parallel Continuous Simulated Tempering (PCST) was used to refine the model. PCST not only improves the model quality but also improves the model geometry.

2.3 Results and Discussion

2.3.1 Generation of monoclonal antibodies

RSV F specific monoclonal antibodies (mAbs) were generated using conventional methods (105). Female BALB/c mice were immunized by interperitoneal injection with RSV F710 antigen. Spleens were collected and single cell suspensions produced from pooled spleens that were depleted of IgM splenocytes by magnetic bead cell sorting. IgG enriched splenocytes were fused with P3X63.Ag.6.5.3 myeloma cells by conventional methods. Hybridomas were screened with an RSV F ELISA and positive cultures cloned by limiting dilution and expanded stepwise to 75 cm² cultures and the antibodies purified from culture medium by protein G affinity chromatography. Five hybridomas producing RSV F specific mAbs were selected for characterization. mAbs R6.29, R1.42 and R6.76 were determined to be IgG1 subclass, R4.C6 be IgG2a, and R6.46 be IgG3.

2.3.2 Purification of RSV F710 wild type and mutant proteins

The Bac-to-Bac® Baculovirus Expression System was used for the protein expression. Three recombinant bacmids of RSV F710 were transfected into Sf9 cells
using Cellfectin™ II. After 90 hours transfection, western blot was used to compare the protein expression level in each transfection supernatant (Figure 2.3 A). The transfection supernatant with the highest protein expression was harvested and amplified.

Before large-scale expression and purification, small-scale expression test was used to decide the best infection MOI. Time course expression test was also performed. Western blot was used for analysis. As shown in Figure 2.3 B & C, the best MOI is 2, and after 48-60 hours of post-infection the expression yield is the highest.

Figure 2.3: Expression test for RSV F710.
(A) Western blot analysis for the expression levels of RSV F710 in recombinant baculovirus by transfection using three bacmids. The middle one has the strongest signal, thus, the corresponding transfection supernatant was harvested and amplified.
(B) Western blot analysis for the expression levels of RSV F710 by using different MOI. MOI = 2 is the optimal one.
(C) Time-course of RSV F710 expression by using MOI = 2. Western blot analysis with sampling at post-infection 48 hrs, 60 hrs, and 72 hrs. Post-infection 48-60 hrs is optimal.

To purify RSV F710 from High Five cells, 1.5 × 10^6 cells/ml cells were infected with the recombinant baculovirus at an MOI of 2 and the cell culture supernatant containing secreted soluble RSV F710 was harvest 48-60 hours post-infection. The
supernatant was dialyzed to remove the small amino acids other supplement components in the cell culture medium, and incubated with Ni-NTA resin. Buffer containing 25 mM Imidazole was used as wash buffer, and that with 250 mM Imidazole was used as elution buffer. Samples were taken for analysis using 12% SDS-PAGE in each purification step, as shown in Figure 2.4 A. The eluted protein solution still had some contaminants, so ion exchange chromatography (Mono S 5/50 GL column) was used to separate RSV F710 with other contaminant proteins. The running buffer contains 20 mM Hepes pH 7.0 and NaCl gradient from 50 mM to 1 M. RSV F710 protein was eluted at conductivity of 30 mS/cm corresponding to 300 mM NaCl (Figure 2.4 B). Peak containing RSV F710 was collected and concentrated for the final size exclusion chromatography step.

The superdex 200 10/300 GL column was used in size exclusion chromatography. There is a major peak shown in the chromatography curve eluted at around 11.2 ml, a position slightly larger than the 150 kD MW standard (Figure 2.4 C). That means the purified RSV F710 is a trimer compared to its monomer’s molecular weight. 12% SDS-PAGE gel result shows the RSV F710 protein is pure enough.
Figure 2.4: Purification of RSV F710.
(A) Ni-NTA affinity purification of RSV F710, analyzed by 12% SDS-PAGE on reducing and non-reducing conditions.
(B) The ion-exchange chromatogram run by using MonoS 5/50 GL column and 12% SDS-PAGE analysis of fractions from the monoS column.
(C) The size exclusion chromatogram run by using Superdex200 10/300 GL column and 12% SDS-PAGE analysis of fractions from the Superdex200 column. Protein standards of known molecular weight are labeled on the base of the chromatogram.
For the RSV F710 mutants, three recombinant bacmids for each mutant (Mutant/SiteII, Mutant/SiteIV, and Mutant/SiteII&IV) were transfected into Sf9 cells using Cellfectin™ II. Western blot was used to compare the protein expression level in each transfection supernatant. For RSV F710 mutant/SiteII, the expression level was pretty low, as weak signals detected in western blot. For RSV F710 mutant/SiteII&IV, the expression level was even lower, as almost no signal detected in western blot. Only RSV F710 mutant/SiteIV has strong signal in western blot, indicating this protein can be expressed and purified. The transfection supernatant with the highest protein expression for RSV F710 mutant/SiteIV was harvested and amplified. Time course expression test was also performed before large-scale expression and purification (Figure 2.5 A).

1.5 × 10^6 cells/ml High Five cells were infected with the RSV F710 mutant/SiteIV baculovirus at an MOI of 2 for 70 hrs and the cell culture supernatant was harvested. The purification procedure is same as for RSV F710 protein. Mono S 5/50 GL column was used to separate the mutant/SiteIV protein from contaminants after Ni-NTA affinity purification. Buffer containing 20 mM Hepes pH 7.0 and NaCl gradient from 50 mM to 500 mM was used. RSV F710 mutant/SiteIV was eluted at conductivity of 28 mS/cm corresponding to 280 mM NaCl. This mutant protein was eluted at 11.4 ml in size exclusion chromatography, a position almost the same as that of RSV F710 (Figure 2.5).
Figure 2.5: Purification of RSV F710 Mutant/SiteIV.

(A) Western blot analysis for the expression levels of RSV F710 Mutants in recombinant baculovirus by transfection using three bacmids for each mutant. The expression level of RSV F710 mutant/SiteII is pretty low. RSV F710 mutant/SiteIV has good expression level. RSV F710 mutant/ SiteII&IV almost has no expression.

(B) Ni-NTA purification of RSV F710 Mutant/SiteIV, analyzed by 12% SDS-PAGE on reducing condition.

(C) The ion-exchange chromatogram run by using MonoS 5/50 GL column and 12% SDS-PAGE analysis of concentrated fraction 24-26 from the monoS column.

(D) The size exclusion chromatogram run by using Superdex200 10/300 GL column and 12% SDS-PAGE analysis of concentrated peak from the Superdex200 column. Protein standards of known molecular weight are labeled on the base of the chromatogram.
2.3.3 Characterization of monoclonal antibody binding specificity and neutralization

An enzyme linked immunoadsorbent assay (ELISA) was used to determine the half maximal effective concentration (EC$_{50}$) of mAbs binding to RSV F710 and to a site II synthetic peptide (RSV F aa 257-277). Antibodies R4.C6, R6.29, R1.42 and R6.76 bound RSV F710 with an EC$_{50}$ = 2-24 ng/mL, which was similar to palivizumab (EC$_{50}$ = 3 ng/mL). mAb R6.46 bound RSV F710 at 10-125-fold higher concentration (EC$_{50}$ = 250 ng/mL) (Table 2.1). Antibody binding to site II peptide was also determined by ELISA. R4.C6 bound the site II peptide at very low concentration (ED$_{50}$= 1 ng/mL), which is 5000-fold lower than palivizumab (EC$_{50}$ = 5000 ng/mL). R6.46 and R6.29 bound site II peptide with EC$_{50}$ = 2450 and 5000 ng/mL, which is comparable to palivizumab. mAbs R1.42 and R6.76 failed to bind the site II peptide (EC$_{50}$ >10 µg/mL) (Table 2.1).

The binding affinity (K$_{d}$) of mAbs to RSV F nanoparticles and site II peptide was determined by surface plasmon resonance (SPR) using a Biacore T200 instrument. R6.46 and R6.29 bound RSV F nanoparticles with high affinity (K$_{d}$= 3.21 nM and 1.59 nM, respectively), which was similar to the binding affinity of palivizumab (K$_{d}$ = 3.38 nM). R6.46 and R6.29 also bound the site II peptide with similar high affinities (K$_{d}$ = 19.6 nM and 29.4 nM, respectively), which was 4-6 fold higher affinity than palivizumab (K$_{d}$ = 125 nM). mAb R1.42 and R6.76 failed to bind the site II peptide, which is consistent with the lack of peptide binding in the ELISA. Interestingly, R4.C6 bound RSV F nanoparticles and the site II peptide at sub-nanomolar concentrations. R4.C6 binding affinity to RSV F nanoparticles was 38-fold greater than palivizumab (K$_{d}$ = 0.087 nM vs.
3.38 nM, respectively) and the binding affinity to site II peptide was 300-fold greater than palivizumab (K_D = 0.4 nM vs. 125 nM, respectively) (Table 2.1).

A plaque reduction assay was used to determine the neutralizing potency of these mAbs. Serially diluted antibodies were combined with RSV/A Long (250-350 TCID_50) and the mixture was added to HEp-2 cells in 96-well micro culture plates for 4-5 days. Infectious virus was determined by staining the cultures with anti-RSV M2-1 mAb. The half maximal inhibitory concentration (IC_{50}) was calculated from the optical density (OD) at 450 nm. mAbs R1.42, R6.46, and R4.C6 neutralized RSV/A Long with IC_{50} = 19.6-1300 ng/mL, which was similar to palivizumab and motavizumab (IC_{50} = 323 ng/mL and 19.6 ng/mL, respectively). R6.29 and R6.76 failed to neutralize RSV/A Long (IC_{50} >10 µg/mL) (Table 2.1).

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^a Antigenic site II synthetic peptide RSV F amino acids 255-276 (NSELLSLINDMPITNDQKKLMSNNV).

^b RSV F NP: RSV fusion glycoprotein nanoparticles.

> : Indicates negative binding to site II peptide or poor neutralization of RSV/A2 (>10,000 ng/mL). n.d.: not done.

Table 2.1: Characterization of RSV F specific monoclonal antibodies.
2.3.4 Passive protection by RSV F specific monoclonal antibodies in mice challenged with RSV/A Long

Protective efficacy of the mAbs was determined in mice challenged with RSV/A. In this study, female BALB/c mice (N = 6/group) were administered 100 µg of purified mAb R4.C6, R1.42, D25 or palivizumab by intraperitoneal injection. A placebo group received PBS. One day following treatment, all mice were challenged intranasally with 105 pfu RSV/A Long. At 4 days post infection, the animals were sacrificed and lung homogenates were prepared and virus load determined with a quantitative plaque assay. Passively treated animals had little or no virus load in the lung samples (LOD 102 pfu/g lung tissue). In contrast, placebo treated animals had elevated virus load in the lung samples 256 pfu/g lung tissue (Figure 2.6).

Figure 2.6: Passive protection against challenge with RSV/A Long in mice. Groups of mice (N = 6/group) were treated with 100 µg purified monoclonal antibody by intraperitoneal injection. The placebo group received phosphate buffered saline. One day later, treated and control animals were intranasally challenged with ~105 pfu RSV/A Long. The right and left lung lobes were collected 4 days post infection and homogenates prepared. Infectious virus titers determined by a quantitative plague reduction assay. The geometric mean titer is indicated by the horizontal bar and the 95% CI is indicated by the
vertical line. The assay limit of detection (LOD) is indicated by the dashed line. Palivizumab (Pali) and R4.C6 (4C6).

2.3.5 Epitope binning of monoclonal antibodies by biolayer interferometry

To further characterize the antigenic sites bound by the mAbs, epitope binning was performed by biolayer interferometry using an Octet OK384 system. Histidine tagged RSV F710 was immobilized on anti-penta-histidine biosensor tips. Captured RSV F710 was exposed to the first antibody and then to the second antibody (analyte antibody), and competitions between antibodies were measured. Palivizumab and RSV14N4 (site IIa), motavizumab and RSV3J20 (site IIb), and RSHZ19 (site IV) were used as controls to define the different antigenic sites. R6.29 and R6.46 both competed the binding of the site IIa and site IIb specific antibodies (Figure 2.7). Although these antibodies both bound site II, R6.46 had neutralizing activity while R6.29 did not neutralize RSV/A Long (Table 2.1), suggesting these antibodies bound site II differently. R6.29 properties are similar to the previously described human mAbs 4B6, 9J5 and 12I1, which bind site II but have no virus neutralizing activity (Mousa, J 2016). R6.29 many bind to the proposed antigenic site VII. Monoclonal antibodies R1.42 and R6.76 competed for the binding to antigenic site IV with RSVZ19. These antibodies target antigenic site IV (Figure 2.7). R1.42 neutralized RSV/A Long while R6.76 had no neutralizing activity, which indicates these antibodies bind site IV differently (Table 2.1).

Among the tested antibodies, R4C.6 broadly competed with the binding of palivizumab/RSV14N4 (site IIa), motavizumab/RSV3J20 (site IIb), R6.29 (site VII) and RSHZ19/R1.42/R6.76 (site IV). These results suggested that R4.C6 uniquely bind to a
previously undefined epitope comprising antigenic sites II/VII and IV (Figure 2.6). Thus, the next step is depicting the binding details of R4.C6 to RSV F710 by structural studies.

![Table](chart.png)

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Figure 2.7: Epitope binning for monoclonal antibody binding to RSF F glycoprotein post-fusion conformations.

Antibody cross-competition determined by biolayer interferometry with an Octet QK384 system. Histidine tagged RSV F710 post-fusion protein was immobilized on an anti-penta-histidine biosensor tips. The data indicate the percent binding of the competing second antibody in the presence of the first antibody, compared with the competing antibody alone. Cells filled with black indicate complete competition and intermediate completion is indicated with light gray cells, if the signal was between 33% and 60%. No competition is indicated by the dark gray cells if the signal was >60%. Antigenic sites IIa/b, VII, IV and II/IV are indicated at top and side colored cells. Cells outlined in red indicate R4.C6 competition with sites II, VII, and IV antibodies. Palivizumab (Paliv), motavizumab (Motav), not determined (n.d.).

2.3.6 Preparation of R4.C6 Fab and RSV F710 complex

To prepare the complex of R4.C6 Fab with RSV F710, purified RSV F710 protein trimer was mix with R4.C6 at different molar ratios (RSV F710 : R4.C6 Fab = 3:3.5; 3:4; 3.5; 3:6). The mixture was incubated on ice for 30 minutes and analyzed by size
exclusion chromatography. The peak positions of different ratios were compared. When using ratio higher than 3:4, the peak positions will keep the same. Thus ratio 3:4 was chosen for the complex preparation. The complex was eluted at volume 9.8 ml, and the excess R4.C6 was eluted as a single peak at volume 16 ml. Compared with the size-exclusion chromatography profile of RSV F710 protein only, the complex peak has 1.5 ml shift in elution volume, shown in Figure 2.8. 12% reduced Bis-Tris SDS-PAGE analysis indicates both RSV F710 and R4.C6 Fab exist in the peak at 9.8 ml. Fractions from this peak were collected and concentrated to 2 mg/ml for storage and was subjected to following cryo-EM specimen preparation.

Figure 2.8: Size-exclusion chromatography profiles of RSV F-R4.C6 complex. Size-exclusion chromatography profiles of RSV F-R4.C6 complex (black solid line curve) and RSV F trimer itself (blue dashed line curve) from Superdex200 10/300 GL column (GE Healthcare). The peaks of RSV F-R4.C6 complex, RSV F trimer, and excess R4.C6 are labeled. Protein standards of known molecular weight are labeled on the base of the chromatogram. Coomassie-stained 12% reduced Bis-Tris SDS-PAGE gel shows RSV F (F1 subunit and F2 subunit) and R4.C6 from the complex peak.
2.3.7 Negative stainning electron microscopy of R4.C6 Fab – RSV F710 complex

Before apply the R4.C6 Fab – RSV F710 complex to cryo-EM analysis, negative staining was used to test the quality of the specimen. **Figure 2.9** shows the raw negative-staining image of purified R4.C6 Fab – RSV F710 complex taken by using JEM2100 with CCD at magnification 60K. From this raw image we can see the specimen is homogeneous and can be subjected to cryo-EM analysis.

![Representative negative-staining image of R4.C6 Fab in complex with RSV F710. Image was taken using CCD camera on JEM2100 at 60,000x magnification.](image)

**Figure 2.9:** Representative negative-staining image of R4.C6 Fab in complex with RSV F710.

Image was taken using CCD camera on JEM2100 at 60,000x magnification.

2.3.8 Cryo-EM and 3D reconstruction for R4.C6 Fab – RSV F710 complex

Because of the unique binding properties of R4.C6 with sites II and IV, we next determined the structural bases for binding of this antibody to these antigenic sites. Purified RSV F-R4.C6 complex was used for single-particle electron cryo-microscopy
(cryo-EM) analysis. By using Graphene oxide covered Quantifoil Copper 200 mesh holy grids, we got the optimized freezing condition with specimen having uniform, thin enough ice thickness and evenly distributed particles. Graphene oxide covered grids are useful with minimized background noise compared with other support film grids. Using this kind of grids can obtain thinner ice thickness, leading to higher resolution images, especially for small molecular proteins. Besides that, preferred orientation problem can be solved.

2734 DDD images were collected using JEM3200 operating at 300 kV and recorded using K2 Summit camera in super-resolution electron counting mode at 30,000x microscope magnification. **Figure 2.10 A** shows the raw electron micrograph of ice-embedded RSV F-R4.C6 complex after motion-correct process. The Fourier power spectrum of the micrograph as in **Figure 2.10 B** shows detail of the raw image in Figure 2.10 A as high as 3.5 Å, indicating the high quality of recorded data, which enables high-resolution 3D reconstruction. From two-dimensional (2D) class averages of particle images we can see different views of the particle, including top view, side view, and tilted view (**Figure 2.11**). Some secondary structural features can also be seen from these 2D classes. This also indicates the high quality of the collected data.
Figure 2.10: Cryo-EM of the purified RSV F – R4.C6 complex.
(A) Representative raw electron image of ice-embedded RSV F-R4.C6 complex on graphene oxide grids recorded using K2 Summit camera. White circles indicate particles from the top view; black circles indicate particles from the side view. Scale bar, 200 Å. The shown image is motion-corrected using MotionCor2. (B) Fourier power spectrum of the micrograph shown in (A) with Thon rings and water ring 3.5 Å labeled.

Figure 2.11: 2D class averages of the RSV F-R4.C6 complex.
(A) Representative 2D class averages of the RSV F-R4.C6 complex obtained by using RELION2.0. C3 symmetry is apparent in these 2D class averages. (B) Euler angle distribution plot of all particles used for the final 3D reconstruction. Bar length and color (blue, low; red, high) is proportional to the number of particles contributing to each specific view. Refined reconstruction map from different angles are also shown.
An initial model was generated with 3-fold symmetry imposition from 2-D reference-free averages using 7,456 manually picked particles, as shown in Figure 2.12. This initial reference map was masked so that only the RSV F part was kept. 234,479 good particles were selected from all the 543,639 particles by several rounds 2D classification and 3D classification. These good particles were used in several rounds of 3D auto-refinement and finally gave near-atomic resolution density map (Figure 2.13 A). This final density map has an overall resolution of 3.9 Å, using gold-standard Fourier shell correlation (FSC) = 0.143 criteria (Figure 2.13 C). From this density map, we can easily recognize a RSV F trimer and three R4.C6 Fab molecules. Resolution is different in various parts of the map (Figure 2.13 B), showing that the center region exhibit better resolvability than the outer region. This difference is probably due to the flexibility in the outer region.

Figure 2.12: Initial model of RSV F-R4.C6 complex.
Initial model was generated from 2-D reference-free averages using 7,456 manually picked particles, C3 symmetry was applied. Left: side view; Right: top view with 90°-rotation outwards.
Figure 2.13: Cryo-EM 3D reconstruction for RSV F-R4.C6 complex and resolution estimation.

(A) Cryo-EM 3D density map of R4.C6 in complex with RSV F. Side view and rotated 90° view are shown.

(B) The cryo-EM density map is colored based on ResMap local resolution estimation. The cryo-EM density map exhibits local resolution variation ranging from 2.7 Å to 4.6 Å, with the most highly resolved densities in the center region, while low-resolution densities in the more flexible outer region.

(C) Gold-standard fourier shell correlation (FSC) curve for the 3D reconstruction generated with RELION2.0, marked with resolution corresponding to FSC = 0.143. After the gold standard map refinement, the final resolution is 3.9 Å.
The post-fusion RSV F (PDB code: 3RRR) and the homology model (PDB code: 1I3G) of R4.C6 Fab structures were docked into the map. The refinement of the whole structure was carried out by Phenix real-space refinement, Coot and PCST. Figure 2.14 shows the Cryo-EM densities of some selected secondary structures of R4.C6 Fab-RSV F complex, indicating the model build and refinement is good. Table 2.2 shows the Cryo-EM data collection and refinement statistics.

**Figure 2.14:** Representative cryo-EM density maps of the R4.C6-RSV F glycoprotein complex.
The cryo-EM densities for selected regions are shown superimposed on the corresponding RSV F-R4.C6 complex model. Map density is in dark mesh. Residue atoms colored C = silver grey, N = blue, O = red, S = yellow.
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Table 2.2: Cryo-EM data collection and refinement statistics
2.3.9 Structure of R4.C6 in complex with RSV F710 post-fusion

The RSV F-R4.C6 complex structure has a RSV F trimer surrounded by three R4.C6 Fab molecules (Figure 2.15). The structure of trimeric RSV F is a cone shape, which has three protomers. Each protomer is composed by F2 subunit and F1 subunit connected by two disulfide bonds. The N- and C- terminal domains of F1 subunit in each protomer together form as a six-helix bundle at the tail. This six-helix bundle is acknowledged as the key characteristic of post-fusion state. Asn70 of F2 subunit and Asn500 of F1 subunit have N-acetyl-D-glucosamine moiety attached.

Figure 2.15: Overall structure of R4.C6 Fab in complex with post-fusion RSV F10. Ribbon representation of the model of R4.C6 Fab bound to RSV F glycoprotein trimer in postfusion conformation. Each F2/F1 monomer has a different color (Cyan, Yellow, and Green). R4.C6 Fab is colored as: Orange: Light Chain; Violet: Heavy chain. Both side view and 90°-rotation outwards view are shown. Glycans at Asn70 and Asn500 are shown as stick.
Only the variable region of the three R4.C6 Fab molecules was modeled, since the density for the constant region is very weak, and what we care about most only involves the variable region. Electron density at the interface between RSV F trimer and each R4.C6 Fab was well defined. Each R4.C6 Fab recognizes a quaternary epitope across two protomers, as shown from the interface (Figure 2.16). The R4.C6 heavy chain (shown in violet ribbon) is facing to one protomer (shown in cyan surface), while the R4.C6 light chain (shown in orange ribbon) is facing to a neighboring protomer.

Four complementarity determining regions (CDRs) in total are involved into the binding interaction. The heavy chain (HC) CDR2 and CDR3 of R4.C6 Fab interact with the α-helix in the antigenic site II motif, with the pivotal hydrogen bonds formed between Asp53 of R4.C6 HC CDR2 and Lys272 of RSV F, Asp 48 of R4.C6 HC CDR2 and Asn268 of RSV F, Asn99 of R4.C6 HC CDR3 and RSV F Asp269 (Figure 2.16). The light chain (LC) of R4.C6 also has interactions with RSV F site II α-helix. Arg30 of R4.C6 LC CDR1 forms hydrogen bonds with Asp263 of RSV F. In addition, there are van der Waal interaction and hydrophobic interaction between Leu50 of R4.C6 LC CDR2 and a loop formed by RSV F Pro265, Ile266, Thr267 between the two α-helices.

The light chain of R4.C6 Fab is in close contact with the antigenic site IV motif spanning RSV F residue 422 to 438 with a high degree of shape complementarity between them. In addition, there are two hydrogen bonds formed between them. On is located between Arg65 of R4.C6 LC and RSV F Asn428; and the other one is between Ser67 of R4.C6 LC and RSV F Lys427. Besides that, Asn454 of RSV F also involved in the binding interaction by forming hydrogen bonds with both Ser100 and Glu101 (Figure 2.17).
Figure 2.16: The interface between RSV F and R4.C6 heavy chain (HC) and light chain (LC).
RSV F710 is shown as yellow surface and cyan surface for two protomers. R4.C6 Fab is shown as ribbon; Orange: Light chain; Violet: Heavy chain. CDRs are labeled for R4.C6 Fab.
Figure 2.17: Detailed interactions between R4.C6 Fab and RSV F antigenic site II. The key residues involved in the interaction are labeled. Hydrogen bonds are shown as dark dashed lines. RSV F Site II is colored as cyan; R4.C6 Fab heavy chain (HC) is colored as violet, and R4.C6 Fab light chain (LC) is colored as orange. Cryo-EM density maps around the interactions are shown in grey mesh.
Figure 2.18: Detailed interactions between R4.C6 Fab and RSV F antigenic site IV. The key residues involved in the interaction are labeled. Hydrogen bonds are shown as dark dashed lines. RSV F Site IV is colored as yellow; R4.C6 Fab heavy chain (HC) is colored as slate, and R4.C6 Fab light chain (LC) is colored as orange. Cryo-EM density maps around the interactions are shown in grey mesh.
2.3.10 Comparison of epitopes recognized by different Site II/IV specific antibodies

hRSV 14N4 and Motavizumab both specifically target antigenic site II of RSV F. The superimposed structure of 14N4-RSV F complex and Motavizumab-RSV F complex reveals these two antibodies bound RSV F in different angles (81). The shifted binding angle of motavizumab away from 14N4 is 42° as reported. The antigenic site II of RSV F is classified as antigenic site IIa and IIb for neutralizing poses, and site VII for non-neutralizing poses. hRSV 14N4 targets site IIb, while motavizumab targets site IIa. R4.C6 bound RSV F through interacting with both site II and site IV. It can compete with the binding of 14N4 (Site IIb), Motavizumab (Site IIa), and R6.29 (Site VII) as shown in Figure 2.6. To compare with the epitopes of these site II specific antibodies, structures of each RSV F-Fab was superimposed at site II motif of amino acids 254 – 277. From Figure 2.19 A and B left panel, we can see R4.C6 binds antigenic site II at a different orientation than 14N4 and Motavizumab. The shifted binding angle of 14N4 away from R4.C6 is 150.2°. And the shifted binding angle of Motavizumab away from R4.C6 is 117°. The differences in binding angle allow R4.C6 to be free of occupying epitope at site IV and interacting with both antigenic sites. Epitopes at site II of 14N4 and R4.C6 are mostly overlapped, while epitopes at site II of Motavizumab and R4.C6 are partially overlapped (Figure 2.19 A and B right panel).

We also compared the epitopes of R4.C6 and 101F Fab by overlaying the antigenic site IV amino acid Asn428 to Asn437 of RSV F. As shown in Figure 2.19 C left panel, 101F binds to RSV F in a completely different angle, with no contact to the site II motif. The shifted binding angle of 101F away from R4.C6 is 161.7°. Interestingly, there isn’t any overlapped residue of RSV F involved in interaction with R4.C6 and
101F. Three residues at RSV F site IV interact with R4.C6. While, six residues of the site IV motif interact with 101F, including Arg429, Ile431, Lys433, Thr434, Phe435, and Ser436. More residues involved in the interaction with 101F may be because the structure was solved using the site IV peptide not the complete RSV F. The peptide, without any other restraints, can adopt a conformation to have more contacts with 101F. As for R4.C6, it has additional interactions with site II, so the contacts with site IV are restricted.
Figure 2.19: Epitopes comparison of R4.C6 with other SiteII and SiteIV antibodies.
For the left panel, RSV F – postfusion is shown as grey surface, while all Fab molecules shown as ribbon. Orange: R4.C6 Fab light chain; Violet: R4.C6 Fab heavy chain; Green: 14N4 Fab; Yellow: Motavizumab Fab; Cyan: 101F Fab.
2.3.11 R4.C6 Fab binding affinity of RSV F710 and its mutants

From the structure of R4.C6 Fab in complex with RSV F710, we found some important residues on RSV F710 that may interact with R4.C6, as T267, N268, D269, and K272 in antigenic site II, and N428, N454 in site IV. Thus we made mutation constructs for RSV F710 harboring mutations T267A/N268G/D269G/K272A at antigenic site II (Mutant/SiteII), mutations of N428G/N454G at antigenic site IV (Mutant/SiteIV), and the mutations at both antigenic sites (Mutant/SiteII&IV). Unfortunately, only RSV F710 mutant/SiteIV can be purified. The binding affinities to R4.C6 Fab of RSV F710 and Mutant/SiteIV, respectively, were measured by using Bio-Layer Interferometry (BLI)-based OCTET RED96 system. The Ni-NTA biosensor tips with RSV F710 or Mutant/SiteIV protein immobilized were subjected to associating with R4.C6 Fab in kinetic buffer (PBS with 0.01% bovine serum albumin) for 20 mintes and dissociating in the kinetic buffer (without R4.C6 Fab) for additional 20 minutes. As from Figure 2.20, the binding trends for R4.C6 of both RSV F710 and Mutant/SiteIV protein are similar, but RSV F710 has slightly stronger binding to R4.C6. The apparent equilibrium dissociation constant (K_d) of RSV F710 with R4.C6 Fab is 0.955 nM, while the K_d of Mutant/SiteIV is 1.31 nM. The binding affinity of Mutant/SiteIV has 30% decrease with R4.C6. That means residues N428 and N454 have significant impact on the R4.C6 binding.
Since RSV F710 Mutant/SiteII cannot be purified, we next sought to test the binding affinity of a series of linear site II peptides with R4.C6 Fab by SPR analysis. Palivizumab was used as a control. Linear site II peptides were produced from the site II full length sequence encompassing RSV F residues 255-NSELLSLINDMPITNDQKMLMSNNV-276 and truncated peptides with amino-terminal and carboxy-terminal deletions. R4.C6 and Palivizumab were immobilized onto a Biacore protein A sensor chip and the chip was exposed to different concentrations of each peptide. R4.C6 bound the full-length peptide with high affinity ($K_D = 0.4$ nM) (Table 2.1) as well as peptides with the first 6-10 aa deleted from the amino-terminus.
R4.C6 failed to bind peptides where the carboxy-terminal aa 275-MSNNV-279 was deleted. These results indicate that R4.C6 binds with high affinity to a linear sequence located near the carboxy-terminal portion of antigenic site II (Table 2.3). In contrast, only the full length of site II has binding to Palivizumab. Furthermore, these results are consistent with observation that R4.C6 binds the site II peptide and RSV F at low concentrations (EC50 = 1 ng/mL and 8 ng/mL, respectively) (Table 2.1).

<table>
<thead>
<tr>
<th>Antigenic site II peptides</th>
<th>R4.C6</th>
<th>Palivizumab</th>
</tr>
</thead>
<tbody>
<tr>
<td>254-NSELLSLINDMPITNDQKKLMSNNV-278</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>254-NSELLSLINDMPITNDQKKL-273</td>
<td>+</td>
<td>&gt;</td>
</tr>
<tr>
<td>260-LINDMPITNDQKKLMSNNV-278</td>
<td>++</td>
<td>&gt;</td>
</tr>
<tr>
<td>254-NSELLSLINDMPITN-268</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>259-SLINDMPITNDQKKL-273</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>264-MPITNDQKKLMSNNV-278</td>
<td>++</td>
<td>&gt;</td>
</tr>
</tbody>
</table>

>: indicates negative binding to antigenic site II peptide

Table 2.3: Binding of R4.C6 Fab to site II synthetic peptides determined by surface plasmon resonance.
2.4 Conclusion

In this chapter, we describe a novel mAb R4.C6 that uniquely binds to antigenic sites II and IV on the RSV F glycoprotein. The mAb was generated in mice immunized with a near full length RSV F glycoprotein NP. Epitope binning by BLI using Octet OK384 instrument revealed that mAb R4.C6 broadly competes for binding with neutralizing humanized and human mAbs targeting antigenic site IIa (palivizumab and hRSV 14N4) and site IIb (motavizumab and hRSV 3J20) as well as non-neutralizing mAb (R6.29) binding to antigenic site VII. Unexpected, mAb R4.C6 also competed binding of neutralizing and non-neutralizing mAbs RSHZ19, R13.6 and R6.76 directed at antigenic site IV. R4.C6 bound RSV F glycoprotein and a site II synthetic peptide with sub-nanomole affinity (KD = 0.087 and 0.4 nM), which was 38- to 300-fold higher affinity than palivizumab. Although R4.C6 binding affinity was significantly greater than palivizumab, its neutralizing potency was 4-fold lower than palivizumab (IC50 = 1300 vs 323 ng mL-1) and ~95-fold lower neutralizing potency than R1.42 or motavizumab. In a passive protection model, however, R4.C6 was found to protect mice against LRTI equal to palivizumab and R1.42. These results indicate that in vitro binding affinity did not appear to correlate with in vitro neutralizing potency, although R4.C6 was equally effective as palivizumab at protection against infection.

Using BLI to assess antibody cross-competition with well characterized mAbs, we were able to determine the antigenic sites bound by R4.C6. This antibody broadly competes binding of both neutralizing and non-neutralizing mAbs directed at sites II, VII and IV. Cryo-EM and 3D reconstruction of images provided insight into the structural bases for the interaction of R4.C6 Fab with this new antigenic site. Three R4.C6 Fab
were found to be in association with a single F trimer. Modeling demonstrated that R4.C6 recognizes a quaternary epitope that involved two protomers of the F trimer. Fine mapping demonstrated that two R4.C6 Fab HC CDRs (CDR2 and CDR3) interact directly with residues Asn268, Asp269, and Lys272 in one α-helix in site II, while R4.C6 Fab LC CDR1 interacts with Asp263 in the other α-helix in site II. In addition, R4.C6 Fab LC CDR2 has hydrophobic interaction with the loop between the two α-helices in site II. The site IV loop on the neighbouring protomer of RSV F interacts with R4.C6 LC and HC through Lys427-Asn428, and Asn454, respectively. A comparison of R4.C6 pose to hRSV 14N4 (site IIa) and motavizumab (site IIb) and 101F (site IV) clearly shows that R4.C6 binds these antigenic sites at an angle that is distinct from these antibodies.

In summary, the F glycoprotein is a major candidate for vaccine development of an effective vaccine for prevention of RSV infection. The F glycoprotein, however, is structurally complex with multiple conformations and numerous antigenic epitopes. The X-ray crystal structures of the RSV F in the pre-F and post-F conformations in complex with various mAbs has provided considerable insight into fine mapping of antigenic determinants on various conformations. The generation of new antibodies needs more comprehensive understanding of RSV F conformations and its epitope determinants. We have used cryo-EM and 3D reconstruction at 3.9 Å resolution to determine structural bases for the interaction of R4.C6 Fab with a novel antigenic site II/IV on the RSV F trimer. This has expanded our understanding for RSV F and can help us design novel antibodies targeting RSV F. Cryo-EM is a powerful tool for identification of other antigenic sites and development of new RSV antibodies and vaccines.
Chapter 3

Cryo-EM Structure of Intermediate RSV F

3.1 Introduction

The RSV F protein is very important for the virus infection. It mediates the fusion of the virus membrane with the host cell membrane. F protein is a very dynamic protein with multiple conformations. It undergoes conformational rearrangement and the free energy released during that process is the driving force needed for fusion of the membranes.

The RSV F protein as a type I transmembrane glycoprotein is synthesized as an inactive F0 precursor with two furin cleavage sites. Prior to fusion of the membranes, RSV F will be firstly cleaved successively at two furin cleavage sites by the furin protease, forming a short F2 subunit and a long F1 subunit covalently linked by disulfide bonds and releasing the Pep27. In this so-called prefusion state, the fusion peptide is buried inside the globular head of the trimeric RSV F. The trimer is anchored to the viral envelop by the transmembrane domain and stabilized by the following three-helix bundle formed by the HRB of each F monomer. The host cell surface nucleolin can act as a receptor for binding to RSV F (114). It was proposed that nucleolin binding to RSV F can trigger a conformational shift of RSV F to release the fusion peptide. The N-terminal of F1, including fusion peptide and HRA, will refold towards the host cell membrane. HRAs will form a three-helix bundle and the fusion peptide will be inserted into the host cell membrane. This partially refold state is called as pre-hairpin intermediate state. Latter,
the C-terminal of F1 will be reorganized. It moves towards to the N-terminal. Thus, the HRB in the C-terminal is brought to close the HRA in the N-term, leading to the formation of the more stable six-helix bundle. This state is called as postfusion state. The free energies during multiple RSV F conformational shifts can overcome the thermodynamic barrier to bring the viral and host membrane into close and mix the membranes to form a fusion pore. **Figure 3.1** shows the RSV F conformational changes during the fusion process.

![Diagram](image)

**Figure 3.1**: Cartoon schematics of different conformations of RSV F during the membrane fusion process.
(A) Prefusion state. In this state, fusion peptide (blue line) is buried inside the globular head.
(B) Pre-hairpin intermediate state. Triggering by binding to the nucleolin receptor on host cell surface, RSV F partially refold to release fusion peptide to insert into host cell membrane. HRA also refold to form a three-helix bundle.
(C) Postfusion state. After complete conformational change, HRB moves towards HRA and brings virus membrane and host cell membrane in close contacts, leading to the membrane fusion and formation of a fusion pore. A six-helix bundle is formed as the characteristic of this state.

The structures of RSV F protein have been elucidated by some research groups. But only the structures of RSV F prefusion and postfusion states have been solved. Two key points of RSV F refolding in the fusion process are clear. However, much more structural information about the conformation of this protein, especially about the intermediate state, is needed to further understand the fusion mechanism. In this chapter, single particle cryo-EM was used to determine the structure of another form of RSV F, called BV2052. From the 5 Å resolution density map, we can see this protein has a head and a three-helix bundle stalk. By comparing with the prefusion and postfusion structure of RSV F, BV2052 is in a different state. The structure information helps us understand the fusion mechanism.

3.2 Materials and Methods

3.2.1 BV2052 Construct and protein purification

The construct, here we named it as BV2052, consists of amino acids 1-574 derived from the RSV A2 F (Genbank Accession No. U63644), but with 10 residues deleted (ΔPhe137-Val146) from the fusion peptide domain (FD) and a mutation of residues 131-136 at furin cleavage site II (KKQKQQ to KKRKRR). The transmembrane domain (TM) and cytoplasmic tail (CT) are also included. BV2052 was constructed into the pOET3 vector downstream of AcMNPV basic (p.6.9) promoter. This vector can
improve the expression of glycosylated secreted protein and is compatible with baculovirus system. Sf9 insect cells were used to express the target protein. Ion exchange chromatography and affinity chromatography (Lentil Lectin Sepharose 4B column) were used for purification. Size exclusion chromatography (Superdex200 10/300 GL) was used as the last step for purification with running buffer 20 mM Hepes, pH 7.0, 150 mM NaCl.

3.2.2 Binding affinity of antibodies to BV2052 by Surface Plasmon Resonance (SPR)

A serious antibodies binding affinities to BV2052 were determined by using surface plasmon resonance carried out on Biacore™ T200 instrument (GE Healthcare, Baltimore, MD). Protein A (Fisher Thermo Scientific, Waltham, MA) was immobilized on CM5 chips through amine-coupling reaction. Protein A was diluted to 100 µg/mL in an acetate buffer (pH4) and injected at a flow rate of 10 µL/min for 10 minutes. The targeted coupling level was 300 response units (RU). Antibodies including Palivizumab, Motavizumab, R6.29, 101F, and R4.C6 were injected over the protein A immobilized CM5 chip at approximately 10 µL/min for 45 seconds. Binding of BV2052 was determined by injecting different concentrations of protein (0, 1.1, 3.3, 10, 30 and 90 nM) over the antibody immobilized chip at 40 µL/min for 180 seconds followed by dissociation for 600 seconds in HBS-EP buffer. The sensorgrams were analyzed by BIAcore kinetics analysis using a 1:1 fitting model to determine the kon and koff rates. Chips were regenerated by injection of 100 mM HCl at 40 µl/min for 45 seconds. The apparent equilibrium constant (K_D) was calculated from ratio of K_on / K_off. Binding affinities of RSV F710 were measured in parallel as comparison.
3.2.3 Negative staining of BV2052

Carbon parlodion-coated 400-mesh Copper grids (PolySciences, Warrington, PA) were glow discharged, followed by overlaid with BV2052 at 0.01 mg/ml for 2 minutes. The grids were washed with buffer and stained with 1% phosphotungstic acid. After air dry, the grids were visualized using Hitachi H-7600 transmission electron microscope (Hitachi High Technologies America, Schaumburg, IL) operating at 80 kV and 245,000x magnification. Images were taken using a 1 K x 4 K CCD camera.

3.2.4 Preparation of Cryo-EM specimen

Specimens were plunge-frozen using Leica EM GP automatic plunge freezer (Leica Microsystems, Vienna, Austria) with its environmental chamber at 22°C and relative humidity at 80%. A 3.2μl aliquot of BV2052 diluted to 0.025mg/ml was applied onto Copper 200 square mesh Quantifoil R1.2/1.3 holey carbon grids (Quantifoil, Jena, Germany) coated with graphene oxide. After 25s adhering, the grid was automatically blotted for 4s from the specimen side with Whatman No. 1 filter paper and immediately plunged from the environmental chamber into liquid ethane. Then the grids were transferred and stored in liquid nitrogen before imaging.

3.2.5 Cryo-EM data collection

All grids were screened on a JEM3200FSC cryo-electron microscope (JEOL, Peabody, MA, USA) operating at 300kV, with energy slit of the in-column filter of 30 eV. Images were recorded using K2 Summit direct electron detector camera (Gatan) in super-resolution electron counting mode at 40,000x microscope magnification.
(corresponding to a calibrated physical pixel size of 0.882 Å). The dose rate is 8 electrons/Å²/sec and 50 frames were acquired in a total exposure time of 10 sec. A total of 3,120 DDD images were recorded.

**3.2.6 Cryo-EM image processing**

The dose-fractionated super-resolution raw image stacks were binned 2 X 2 by Fourier cropping resulting in a pixel size of 0.882 Å for further image processing. Each image stack was subjected to motion correction using MotionCor2, the whole frames (all 50 frames) of each image stack were used. Gctf was used to estimate the contrast transfer function parameters. A total of 111,631 particles were boxed out manually using ‘e2boxer.py’ in EMAN2 from all the 3,120 micrographs. Those particles were binned 2 X 2 to increase contrast for following ‘2D classification’ in RELION2.0. Based on the reference-free 2D class averages, good particles were chosen for further process. Several 2D classes were chosen to generate the initial map with 3-fold symmetry imposed using EMAN2. This initial reference map was low pass filtered to 60 Å resolution as a starting point to further process in RELION2.0. Several rounds of iterative 3D classification and 3D auto-refinement were performed. 62,906 particles were used in the final refinement to achieve a 5 Å resolution density map. A soft mask in RELION post-processing was applied before computing the FSCs. The final resolution was estimated by 0.143 cutoff of FSC. The density map was sharpened by applying a B-factor of -250 Å² estimated by an automated procedure.
3.3 Results and Discussion

3.3.1 Purification of BV2052

BV2052 was expressed using Sf9 cells. Purification was performed using lectin affinity and ion exchange chromatography. Finally, size exclusion chromatography (Superdex 200 10/300 GL column) was performed. There was a single peak on the chromatogram, with molecular weight larger than 158 kDa compared with the MW standards, indicating the BV2052 is a trimer (as shown in Figure 3.3). Fractions were analyzed using 12% SDS-PAGE under reducing and non-reducing conditions. The protein was purer enough and was concentrated to 5 mg/ml for storage and following experiment. The F1 subunit plus F2 subunit is smaller than 60 kDa, which is smaller than expect (should be larger than 63 kDa). Thus, mass spec was used to determine the amino acid sequence of this secreted BV2052. The result indicates the secreted protein has been cleaved during the protein process, composing amino acids 26-502 of RSV F / A2 strain, with theoretical MW 52 kDa (the MW of glycans are not included). Compared with RSV F710 (amino acids 26-526), BV2052 lacks the C-terminal 24 amino acids. Figure 3.2 shows the schematics of designed protein and the final purified secreted product.
Figure 3.2: Schematic shown the designed BV2052 and the final purified protein.
(A) The designed BV2052 consists of amino acid 1-574 from the RSV A2 F, composing signal peptide (SP), F2 subunit, F1 subunit, transmembrane domain (TM) and cytoplasmic tail (CT). The second furin cleavage site is mutated as KKQKQQ to KKRKRR, so p27 is still included in the F1 subunit. 10 amino acids (ΔPhe137-Val146) were deleted in the F1 N-terminal fusion peptide domain (FD).

(B) The final purified protein contains amino acid 26-502 as confirmed by Mass Spec. The transmembrane domain, the cytoplasmic tail, and the last 24 amino acids of the C-term of F1 were degraded. The F2 and F1 subunits are connected by two disulfide bonds.

Figure 3.3: Size-exclusion chromatography profile of BV2052.
Size-exclusion chromatography profile of BV2052 from Superdex200 10/300 GL column (GE Healthcare). Protein standards of known molecular weight are labeled on the base of the chromatogram. Coomassie-stained 12% reduced Bis-Tris SDS-PAGE gel shows the concentrated BV2052 peak sample reducing (Left lane: F1 subunit and F2 subunit) and non-reducing (Right lane: F1+F2).
3.3.2 Binding affinity of antibodies to BV2052 by Surface Plasmon Resonance (SPR)

The binding affinity of mAbs to BV2052 and RSV F710 was determined by surface plasmon resonance (SPR) using a Biacore T200 instrument. As from Figure 3.4, Palivizumab bound BV2052 with high affinity, similar to the binding affinity to RSV F710 ($K_D = 0.3063$ nM and $0.2347$ nM, respectively). Motavizumab bound BV2052 with even higher affinity ($K_D = 0.0002136$ nM), which is 100 fold higher than the affinity of Motavizumab to RSV F710. The binding affinity of R6.29 to BV2052 is 1000 fold higher than that to RSV F710 ($K_D = 0.000459$ nM vs. $0.4485$ nM). As for 101F and R4.C6, the affinities to BV2052 are 2 fold higher than that to RSV F710 ($K_D = 0.1665$ nM and $0.444$ nM to BV2052, respectively; $K_D = 0.3447$ nM and $0.9289$ nM to RSV F710, respectively). Overall, these antibodies, except Palivizumab, bind BV2052 with higher affinities than RSV F710.
Figure 3.4: Binding kinetics of antibodies to BV2052 and RSV F710.
(A) Top: Binding curves of antibodies to BV2052. Down: Binding curves of antibodies to RSV F710. The concentrations of BV2052 and RSV F used in the binding experiment are 1.1, 3.3, 10, 30 and 90 nM.
(B) Binding kinetics of antibodies to BV2052 and RSV F710, respectively. K\textsubscript{D}: Apparent equilibrium dissociation constant calculated as Koff/Kon by using 1:1 fitting model; Kon: association rate from the association curves; Koff: dissociation rate from the dissociation curves.

3.3.3 Negative staining electron microscopy of BV2052

Before applying BV2052 to cryo-EM, negative staining was first used to check the quality of specimen. Figure 3.5 shows the raw negative-staining image of purified BV2052 taken by using Hitachi H-7600 TEM with CCD at magnification 245,000x. From this raw image we can see the overall shape of BV2052 is different from RSV F710 and the specimen is good and can be subjected to cryo-EM.
3.3.4 Cryo-EM and 3D reconstruction for BV2052

As from the negative staining image, the overall shape of BV2052 is different from RSV F710 postfusion. To determine the structure of BV2052, single particle cryo-EM was used. Purified RSV F was applied to Graphene oxide covered Quantifoil Copper 200 mesh holy grids. Freezing conditions were optimized for uniform and thinner ice thickness as well as the distribution of particles with optimal concentration. 3120 DDD images were collected using JEM3200 operating at 300 kV and recorded using K2 Summit camera in super-resolution electron counting mode at 40,000x microscope magnification. **Figure 3.6 A** shows the raw electron micrograph of ice-embedded BV2052 after motion-correct process. The Fourier power spectrum of the micrograph as in **Figure 3.6 B** indicates the high quality of recorded data.
Figure 3.6: Cryo-EM of the purified BV2052.
(A) Representative raw electron image of ice-embedded BV2052 on graphene oxide grids recorded using K2 Summit camera at 40,000x magnification. Scale bar, 200 Å. The shown image is motion-corrected using MotionCor2.
(B) Fourier power spectrum of the micrograph shown in (A) with Thon rings and water ring 4 Å labeled.

Figure 3.7 shows the two-dimensional (2D) class averages of particle images analyzed by using RELION2.0. Both side views and top views are included. In addition, we can see some secondary structure elements from the first few classes; that means the quality of data is high. We generated an initial model using EMAN2 and selected 2D classes, as shown in Figure 3.8, C3 symmetry was used as BV2052 forms a trimer.

This initial reference map was low pass filtered to 60 Å resolution as a starting point to further process in RELION2.0. 62,906 particles were selected after several rounds of 2D classification and 3D classification. These good particles were used in several rounds of 3D auto-refinement and finally gave a density map of 5 Å resolution according to the gold-standard Fourier shell correlation (FSC) = 0.143 criteria (Figure 3.9).
Figure 3.7: Representative 2D class averages of the purified BV2052 obtained by using RELION2.0.

Figure 3.8: Initial model generated from 2-D reference-free class averages. C3 symmetry was applied. Left: side view; Right: top view with 90°-rotation outwards.
Figure 3.9: 3D reconstruction of BV2052.
(A) 5 Å resolution density map of BV2052. Side view and rotated 90° view are shown.
(B) Gold-standard fourier shell correlation (FSC) curve for the 3D reconstruction generated with RELION2.0, marked with resolution corresponding to FSC = 0.143.
3.3.5 Comparison of BV2052 with RSV F pre-fusion and post-fusion

To compare BV2052 with the post-fusion RSV F, we generated the density map from the crystal structure of post-fusion RSV F (PDB: 3RRR) composing amino acids 26-502 at 5 Å resolution using Chimera. By superimposing these two density maps, we can see there is dramatic difference between them. As shown in Figure 3.10, the grey density map is BV2052 and the yellow one is post-fusion RSV F. They both have cone-shaped overall shapes, but the head of BV2052 is bigger and the stalk is shorter than that of post-fusion RSV F. The key characteristic of post-fusion RSV F is a six-helix bundle stalk forming by the rearrangement of HRA and HRB. But for BV2052, there is only a three-helix bundle as the stalk. From the Figure 3.11, we can see HRA can fit with the three-helix bundle density of BV2052, but HRB cannot fit any density of BV2052. The second structure elements of BV2052 and RSV F post-fusion at the head part are also different. As for the α-helix Leu78-Met97 and the β-sheets Tyr53-Glu60, Try286-Pro304 of RSV F post-fusion, the densities at the according positions of BV2052 is different (Figure 3.12). It’s not hard to tell that the structure of BV2052 is different from RSV F post-fusion.
Figure 3.10: Superimposed density maps of BV2052 and RSV F post-fusion.
Superimposed density maps of BV2052 (Grey) and RSV F post-fusion (Yellow, generated from PDB 3RRR). The overall shapes of BV2052 and RSV F post-fusion are similar, like a cone-shape, with a head and a stalk. The stalk of RSV F post-fusion is a six-helix bundle, while the stalk of BV2052 is shorter and is a three-helix bundle.
Figure 3.11: Comparison of the stalk part of BV2052 and RSV F post-fusion.
(A) Superimposed density maps of BV2052 (Grey) and RSV F post-fusion (Yellow) with HRB of postfusion been highlighted as red.
(B) Cross-section view before the stalk part. Top: superimposed density map. Middle: RSV F post-fusion density map, HRB is colored as red, HRA is colored as yellow. Down: BV2052 density map shown in grey color. BV2052 can fit with HRA in RSV F post-fusion.
(C) Cross-section view in the middle part of the stalk. Top: superimposed density map. Middle: RSV F post-fusion density map, HRB is colored as red, HRA is colored as yellow. Down: BV2052 density map shown in grey color. BV2052 can fit with HRA in RSV F post-fusion.
Figure 3.12: Comparison of the secondary structure elements in head part of BV2052 and RSV F pre-fusion.
(A) Superimposed density maps of BV2052 (Grey) and RSV F post-fusion (Yellow) with α-helix Leu78-Met97 been highlighted as Cyan and the β-sheets Tyr53-Glu60, Try286-Pro304 been highlighted as green.
(B) Zoomed RSV F post-fusion head part density map and the corresponding structure in cartoon representative.
(C) Zoomed RSV F post-fusion head part density map.

We also compared BV2052 with RSV F pre-fusion. The density map was generated from the crystal structure of pre-fusion RSV F (PDB: 4JHW) composing amino acids 26-502 at 5 Å resolution using Chimera. The overall shapes of BV2052 and RSV F pre-fusion are dramatically different (Figure 3.13). RSV F pre-fusion looks much more compact. RSV F is a very dynamic protein; as it undergoes conformational change from prefusion to postfusion by refold the HRA and HRB. Figure 3.14 shows the superimposed density map of BV2052 and RSV F pre-fusion with HRA and HRB been colored highlighted. We can see the HRA region of prefusion cannot fit any density of BV2052. We may hypothesize HRA in prefusion refolded to form the stalk three-helix
bundle in BV2052. BV2052 is an intermediate state between prefusion and postfusion state.

Figure 3.13: Superimposed density maps of BV2052 and RSV F pre-fusion.
Superimposed density maps of BV2052 (Grey) and RSV F pre-fusion (Pink, generated from PDB 4JHW).
Figure 3.14: Comparison of the BV2052 and HRA region of RSV F pre-fusion.
(A) Superimposed density maps of BV2052 (Grey) and RSV F pre-fusion (HRB is highlighted as Red; HRA is highlighted as Blue; the other part is as Pink color).
(B) (C): Cross-section views at HRA region of RSV F prefusion. Top: superimposed density map. Middle: RSV F pre-fusion density map, HRA is colored as blue. Down: BV2052 density map shown in grey color.
3.3 Conclusion

In this chapter, we performed single particle cryo-EM analysis for secreted BV2052, a truncated RSV F protein as revealed by Mass Spec result. The 5 Å resolution density map is not good enough for 3D reconstruction, but some features can be seen from the density map. The overall shape of this protein is completely different from the prefusion RSV F. Like the postfusion RSV F, it has a head and a stalk. But the stalk part is shorter and characterized by a three-helix bundle, not the six-helix bundle. BV2052 is in a state different from the prefusion and postfusion states. The density map of this three-helix bundle intermediate state can help us understand the fusion mechanism of RSV F.
Note

1. Chapter 2 is based on work contributed to a jointly authored manuscript:

Reference:

Grosfeld, H., Hill, M. G. & Collins, P. L. RNA replication by respiratory syncytial virus (RSV) is directed by the N, P, and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. *Journal of virology* **69**, 5677-5686 (1995).


Bermingham, A. & Collins, P. L. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA


35 Tran, T. L. et al. The nine C-terminal amino acids of the respiratory syncytial virus protein P are necessary and sufficient for binding to ribonucleoprotein complexes in which six ribonucleotides are contacted per N protein protomer. The Journal of general virology 88, 196-206 (2007).


Bermingham, I. M., Chappell, K. J., Watterson, D. & Young, P. R. The Heptad Repeat C Domain of the Respiratory Syncytial Virus Fusion Protein Plays a Key Role in Membrane Fusion. *Journal of virology* **92** (2018).


Patel, N. et al. in *RSV Vaccines for the World Conference* (Malaga, Spain, 2017).


