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ORIGINAL ARTICLE

Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy—a review

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Abstract Infection of infants in their first year of life, children and elderly people with the respiratory syncytial virus (RSV) endangers the life of the patient. An attempt to develop a formalin-inactivated RSV (FI-RSV) vaccine during the 1960s resulted in an aggravated infection in immunized children, leading to hospitalization, while infection of non-immunized children produced much milder symptoms. The reason for this remained an enigma, one which was gradually solved over the last decade by many researchers who studied the molecular biology of RSV infection of respiratory ciliary cells. Clinical studies of RSV-infected patients indicated increased levels of Th2 cytokines and IgE in the patients' sera, suggesting that an allergy-like condition developed during infection. The biomarkers of allergy caused by endogenous or environmental allergens include a marked increase of the Th2 cytokine IL-4 and IgE non-neutralizing antibodies to the allergen. The way allergens trigger allergy was deciphered recently, and will be discussed later. Studies of RSV infection led to the suggestion that RSV patients suffer from allergy prior to RSV infection, a concept that was later abandoned. Studies on HIV-1 [Y. Becker, Virus Genes **28**, 319–331 (2005)] research led me to the hypothesis that since HIV-1 infection induces a marked increase of IL-4 and IgE in serum, an allergy-like condition, the AIDS stage is the result of an allergen motif that is embedded in the shed viral gp120 molecules. It is hypothesized that the viral-soluble G glycoprotein (sG) contains a T cell superantigen (Tsag) that is capable of binding to the V_H3 domain of IgE/FcϵRI$^+$ hematopoietic cells, basophils, mast cells and monocytes, similar to the case of allergens, and that this aggregation causes these innate system cells to degranulate and release large amounts of Th2 cytokines (IL-4, IL-5, IL-10, IL-13) into the blood. The way these Th2 cytokines skew the Th1/Th2 balance toward Th2 > Th1 will be discussed. The aim of the present review is to base RSV pathogenicity on the numerous very good analyses of the virus genes and to suggest a therapeutic approach to treatment that is directed at preventing the inhibitory effects of Th2 cytokines on the adaptive immune system of the patients, instead of inhibiting RSV replication by antivirals. The review of the molecular research on the role of the viral fusion (F) and attachment (G) glycoproteins of RSV provided information on their role in the virus infection: early in infection the F glycoprotein induces Th1 cells to release the Th1 cytokines IL-2, IL-12 and IFN-γ to activate precursors CTLs (pCTLs) to become anti-RSV CTLs. The G and sG glycoproteins attach to FKNR1$^+$ ciliary respiratory epithelial cells as well as directly to eosinophils to the lungs. The sG T cell antigen can also induce the release of large amounts of Th2 cytokines from CD4$^+$ T cells and from FCϵRI$^+$ mast cells, basophils and monocytes. By comparison to HIV-1 gp120 it is possible to show that in the G and sG proteins the T cell antigen resembles the CD4$^+$ T cell superantigen (=allergen) domain of HIV-1 gp120 which aggregates with IgE/FCϵRI$^+$ hematopoietic cells. The increased IL-4 level in the serum inhibits the adaptive immune response: IL-4Rα^+ Th1 cells stop Th1 cytokine synthesis and

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IL-4Rα^+ B cells stop the synthesis of antiviral IgG and IgA and switch to IgE synthesis. In addition, the hematopoietic cells release histamine and prostaglandin which induce wheezing. The gradual increase of sG molecules creates a gradient of fractalkine (FKN) which directs IL-5-activated eosinophils to the lungs of the patient.

Keywords RSV · Pathogenicity genes · sG glycoprotein · CX3C fractalkine domain · Superantigen domain · Allergy · Eosinophilia · Bronchiolitis · Cytokines and antagonists

Introduction

A recent study by Fleming et al. [1] compared the mortality of infants (age group 1–12 months) from RSV and influenza infections in England over the winters 1989/1990 to 1999/2000. The RSV-attributed deaths in infants 1–12 months-old exceeded those of influenza every year except 1989/90 (8.4 deaths and 6.7 deaths per 100,000 population, respectively). The authors summed up the impact over the 11 winters studied and concluded that ''compared to influenza, RSV is associated with more deaths in infants aged less than 12 months''.

The severity of RSV disease in infants and children led Chanock and Parrott [2] to develop a vaccine containing formalin-inactivated RSV (FI-RSV) that was propagated in monkey kidney cell cultures and concentrated to a 100-fold concentration. The RSV vaccine was administered intramuscularly to children residents of Harrison and Arthur Cottages in Junior Village, a District of Columbia Welfare Institution for homeless, but otherwise normal, infants and children. No significant local or systemic vaccine reactions were observed [3]. The RSV vaccine produced high levels of complement-fixing (CF) antibodies and virus-neutralizing antibodies in 27/28 children. Nine months after the initiation of the vaccine study, an acute RSV infection occurred and virus was isolated from 60/141 infant and child residents. The authors noted that the virus infection was significantly associated with febrile pneumonia illness and that ''the vaccine not only failed to offer protection but also induced an exaggerated, altered clinical response to naturally occurring RSV infection in 9/13 vaccinated versus 4/47 non-vaccinated infants. This and additional studies by Chanock's group [4] revealed the marked difference between the successful Salk formalin-inactivated poliovirus vaccine and the unsuccessful RSV inactivated vaccine. The reasons remained an enigma throughout the last 40 years.

Openshaw et al. [5] indicated that the outcome of the FI-RSV vaccination was that 80% of RSV-vaccinated infants were severely infected by RSV and required hospitalization and two infants died. The RSV infection of the vaccinated infants caused an intense inflammatory cellular infiltrate in the lungs, comprising mononuclear cells, eosinophils and polymorphonuclear cells. The sera of the vaccinated infants contained antibodies to the viral fusion protein which lacked neutralizing properties [6], most probably IgE, as suggested by Welliver et al. [7, 8]. These authors reported that RSV-specific IgE antibodies and histamine were found in nasopharyngeal secretions of RSV-infected children [7], suggesting that ''the demonstration of a defect in suppressor cell numbers and function in patients with bronchiolitis due to RSV, which may explain both the exaggerated lymphoproliferative responses to RSV antigen, as well as the overproduction of anti-RSV IgE among these patients''. Most important was the authors' statement that ''similar defects in histamine-induced suppressor-cell function have been demonstrated in patients with asthma and other atopic disorders''. In a later study, Welliver [9] presented two hypotheses on the immune response theories regarding RSV-induced wheezing and asthma. The first hypothesis suggested that the severity of RSV infection is related to the induction of Th2-stimulated immune response at the time of RSV infection, rather than Th1 immune stimulation, indicating ''that the Th2 pathway is at least partially responsible for the development of wheezing and asthma after RSV infection''. However, ''infants with severe hypoxic bronchiolitis appeared to have a more balanced Th1/Th2 response after RSV infection''. The second hypothesis suggested that chemokines may be responsible for the development of severe airway disease. Macrophage inflammatory protein-1 alpha (MIP-1-alpha) is a potent chemokine that attracts high concentrations of eosinophils to the lungs of individuals with hypoxic bronchiolitis.

The outcome of the FI-RSV vaccine study revealed that investigations on the molecular biology of RSV and the host immune response to the virus need to be deciphered before an effective vaccine can be developed. Four decades had already passed and marked developments in molecular virology and immunology made possible the understanding of the relationship between RSV infections and the host adaptive immune response. The present study attempts to review the findings and ideas on RSV pathogenicity and to provide an analysis of the mechanisms by which RSV proteins subdue the patient's adaptive immunity.

RSV infection is the cause of allergy or allergy predisposes children to RSV

Sigurs et al. [10] prospectively compared 47 children hospitalized with RSV bronchiolitis in infancy and 93 healthy children at the mean age of 1 and 3 years. It was reported that the frequency of asthma and allergic sensitization were both significantly higher in the group of RSV-infected children. The same group of children was studied at age 7.5 years and it was reported that ''RSV bronchiolitis has by far the highest independent risk ratio for asthma than a family history of atopy/asthma and also a significantly elevated risk ratio for allergic sensitization. These findings support the theory that RSV influences the mechanisms involved in the development of asthma and allergy in children''. However, Sims et al. [11] reported that ''atopy does not predispose to RSV bronchiolitis or post-bronchiolitis wheezing''. In their analysis of the role of viruses in asthma Message and Johnston [12] quoted the study by Sigurs et al. [10] and indicated that Welliver [13] had failed to demonstrate a relationship between RSV infection and asthma. The authors concluded that it is unclear whether respiratory viruses actually cause asthma.

Peebles et al. [14] reviewed and analyzed seven clinical studies on RSV-infected infants and children with bronchiolitis and allergic disease during infancy. The results of these studies led the authors to conclude that ''the question as to whether severe RSV infection early in life heightens the allergic phenotype and predisposes to asthma later in life as opposed to underlying allergic inflammation predisposes to severe RSV-induced respiratory infection in young children remains unanswered''.

Mosmann et al. [15] and Mosmann and Sad [16] reported that the dendritic cells (DCs), members of the innate cell system, the professional presenters of viral antigen to naïve $CD4^+$ T cells in lymph nodes, polarize the naïve $CD4^+$ T (Th_o) cells into two $CD4^+$ T cell subclasses. Antigen presentation by DC HLA class I polarize naïve T cells to become $CD4^+$ T helper 1 (Th1) cells. Antigen presentation by DC HLA class II molecules polarize naïve $CD4^+$ T cells to become $CD4^+$ T helper 2 (Th2) cells. The Th1 cells produce the cytokines IL-2, IL-12 and IF-γ, inducers of of $CD8^+$ T cell precursors to become cytotoxic T cells (CTLs), and Th2 cells produce the cytokines IL-4, IL-5, IL-6 and IL-10. Welliver et al. [7] reported, 25 years ago, that RSV infection of infants and children causes increased serum IgE and histamine levels in the nasopharyngeal secretions. The relationship between increased IgE and histamine in the respiratory tract during RSV infection will be discussed later.

RSV infection causes increased Th2 cytokines and IgE levels in the sera of RSV-infected children

Roman et al. [17] tested the hypothesis that the increase of virus-specific IgE in RSV-infected children might be a consequence of the increase in Th2 cytokines and quantified the IFN-γ and IL-4 content in the supernatants of peripheral blood mononuclear cells (PBMC) cultured *in vitro* for 24 and 48 h in the presence and absence of phytohemagglutinin and pokeweed mitogen. Cell subsets were obtained from 15 hospitalized infants during an acute RSV lower respiratory infection and from 17 healthy control infants, 1–15 months of age. It was reported that RSV infection increased the number of B cells, decreased the number of $CD8^+$ T cells and activated $CD8^+/CD25^+$ suppressor/cytotoxic T cells. In RSV-infected infants IFN-γ production was subtotally suppressed, whereas IL-4 production was decreased to a lesser degree, giving significantly increased IL-4/IFN-γ ratio compared to the control infants. The author concluded that ''this effect would favor a Th2-type humoral response'' that ''could explain the inflammatory response and the IgE-induced response, both specific and non-specific''.

Aberle et al. [18] used semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) to determine IFN-γ mRNA in lymphocyte subsets from RSV-infected infants using cytometry. It was reported that significantly lower IFN-γ levels and T lymphocyte counts were found in the acute phase of illness in infants with severe RSV disease than in those with a milder clinical course of illness. The induction of RSV-IgE was not related to IFN-γ levels in the acute phase of illness, but rather correlated with IFN-γ expression during convalescence. The authors concluded that the reduced IFN-γ expression may be an important factor in the pathogenesis of severe RSV disease.

Sung et al. [19] hypothesized that the immunological responses of infants to RSV infection (bronchiolitis) and influenza A (upper respiratory tract infection) are different. The authors obtained sera and nasopharyngeal aspirates (NPA) from infants with a coryzal illness, with or without wheeze, who were admitted to pediatric wards during 1998. Cytokines, adhesion molecules, RANTES, IgE and eosinophil cationic protein (ECP) were measured by enzyme-linked immunosorbent assay or fluorescence enzyme immunoassay. RSV and influenza A were diagnosed by virus culture and immunofluorescence. Of the 39 infants studied, RSV infection was confirmed in 11 patients and influenza A in 10 patients. It was found that the serum concentrations of IL-4 and IL-5, RANTES and adhesion molecule 1 in RSV-infected infants were

significantly higher than in those with influenza A. The content of tumor necrosis factor alpha (TNF-α) in the nasopharyngeal aspirates was significantly lower in RSV-infected patients. The authors concluded that ''a predominant Th2 cytokine was observed in RSV infection whereas a predominant pro-inflammatory cytokine response was observed in influenza A-infected infants''.

Pala et al. [20] determined the long-term correlates of infantile bronchiolitis, IL-4 and IFN-γ responses to a panel of antigens in a cohort of 7–8-year-old children with a history of severe RSV bronchiolitis in infancy. Peripheral blood lymphocytes were obtained from 37 hospitalized children with RSV bronchiolitis in infancy and 69 age-, sex- and location-matched control children. The lymphocytes were stimulated *in vitro* with RSV, house dust mite, birch and cat allergens. Cellular proliferation and production of IFN-γ and IL-4 were measured by enzyme-linked immunoSPOT. The ex-bronchiolitis children's T cells produced IL-4 and IFN-γ in response to RSV and cat antigen. Similarly, the control children's T cells responded to RSV and the aeroallergens with IL-4 and IFN-γ. The authors concluded that ''RSV bronchiolitis in infancy may increase the risk of allergic sensitization''.

Legg et al. [21] examined the *in vivo* immune response in 88 babies, with at least one parent with atopy and asthma, throughout their first winter. RSV infection was detected in 28 children with an upper respiratory infection of whom 9 developed bronchiolitis. Nasal lavage specimens were assayed for IFN-γ, IL-4, IL-10 and IL-12 mRNA extracted from stimulated mononuclear cells. It was reported that IL-4/IFN-γ ratio for infants with acute bronchiolitis was elevated in nasal lavage fluid on days 1–2 and days 5–7 of the illness compared to children with upper respiratory tract infection. The IL-10/IL-12 ratio on days 1–2 was higher and IL-18 mRNA levels were reduced. It was concluded that excess type 2 and/or deficient type 1 immune responses play a role in RSV pathogenesis.

Choi et al. [22] investigated the genetic basis for the severity of RSV disease, linked to variants of three Th2 cytokine genes, IL-4, IL-13 and IL-5, clustered on chromosome 5q31.1, in 105 children hospitalized with severe RSV infection and 315 healthy controls. The authors reported that common IL-4 haplotype at five loci included the –589T promoter variant. The pattern was over-represented in patients with severe RSV disease. This promoter was associated with increased IL-4 transcription in patients with asthma. The authors concluded that ''since an altered balance between the Th1 and Th2 immune response has been identified as a critical component in severe RSV disease, further genetic association studies should target additional candidate genes derived from the Th1 and Th2 cytokine pathways, including receptors and signal transducers''.

Van der Sande et al. [23] studied a cohort of children with severe RSV infection during the first year of life (n = 66) and healthy controls (n = 122) and during a 5-year follow-up. The authors tested for immediate hypersensitivity to common allergens, airway reactivity, serum IgE concentration and production of IFN-γ, IL-5 and IL-13 by activated lymphocytes. It was reported that patients with severe RSV disease produced significantly higher IL-13 concentrations in response to tuberculin, had similar skin response to allergens, airway reactivity and serum IgE concentrations. The authors concluded that ''severe RSV infection in early life was associated with persistent increase in the production of type 2 cytokines, which could play a role in the pathogenesis of RSV infection and could influence the immune response to other pathogens''.

Wilson et al. [24] investigated the role IL-10 plays in RSV disease by studying the effects that variations in the IL-10 gene has on the outcome of the disease. Eight single nucleotide polymorphisms (SNPs) spanning the IL-10 gene were selected and haplotypes were constructed. SNPs that efficiently tagged these nucleotides were then typed in 580 infants with severe RSV bronchiolitis and in 580 control subjects. It was reported that none of the haplotypes were associated with RSV bronchiolitis. However, two SNPs (IL-10-1117 and IL-10-3585) were associated with the need for mechanical ventilation.

These studies demonstrated that the Th1/Th2 cytokine balance in the serum of children with severe RSV disease was skewed toward Th2 cytokines. The biomarker for enhanced Th2 cytokines is the reported increase in IgE, IL-5, IL-10 and IL-13 in the sera of RSV-infected children. Yet, increased in IgE content in the children's sera is mediated by a marked increase in IL-4 content in the blood, as was reported for HIV-1-infected people [25, 26]. In these studies it was concluded that the HIV-1 glycoprotein gp120 molecules, which are shed from HIV-1 virions, have superantigen domains which resemble allergens that interact with IgE V_H3 bound to Fc epsilon receptor I^+ ($Fc\epsilon RI^+$) hematopoietic cells (mast cells, basophils and monocytes). After attachment of the viral allergen the hematopoietic cells release large amounts of the Th2 cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 from preformed cytoplasmic granules [27]. The possible role of the RSV soluble G (sG) glycoprotein in the increased levels of IgE and histamine [7] and the skewing of the Th1/Th2 cytokine balance during RSV infection will be discussed below.

The structural and non-structural RSV proteins and their functional domains which are responsible for viral pathogenicity

RSV G glycoprotein: the soluble G (sG) and the virion membrane-bound G (mG)

RSV is a member of the genus Pneumovirus, Pneumovirinae subgroup of the *Paramyxoviridae* family of viruses. RSV virions are membrane-enveloped capsids and possess a single-stranded negative strand RNA genome containing 10 genes which, in infected cells, are transcribed into positive mRNA molecules [28]. The virion envelope contains three glycosylated viral proteins: the surface glycoprotein (G glycoprotein), the fusion protein (F glycoprotein) and the short hydrophobic (SH) glycoprotein. The virion membrane-bound G glycoprotein (mG) molecules bind to cellular receptors on cells (heparin and fractalkine receptors). Following the attachment of virions to cells, the viral trimeric F glycoprotein molecules fuse the virion's envelope to the cellular membrane. These two glycoproteins are the focus of the present review. Although HIV-1 is a lentivirus and RSV is a paramyxovirus, the G and F glycoproteins of both viruses use a similar mechanism of insertion of the viral nucleocapsid into their different target cells. Both viruses use soluble viral glycoprotein: HIV-1 sheds the viral gp120, while RSV-infected cells release one-sixth of the synthesized viral G glycoprotein molecules in a soluble form (sG glycoprotein) from the infected cells. Both viruses are syncytium-forming viruses.

The molecular properties of RSV G glycoproteins

Hendricks et al. [29] reported that the sG glycoprotein is shed from RSV-infected HEp-2 cells at 6 h p.i., prior to virion release. These sG glycoproteins are shorter than the virion's mG glycoprotein, which is 6–9 kDa larger, depending on the virus isolate. The 74 KDa sG protein is released in a non-glycosylated form from RSV-infected cells which were treated with tunicamycin. Sequencing of the RSV sG protein revealed that the enzymatic cleavage site is between residues 65 and 66 and residues 74 and 75, suggesting the presence of two different forms which lack the transmembrane domain of the mG glycoprotein. The virion membrane-bound (mG) protein has a backbone of about 32.6 KDa and contains both *N*- and *O*-linked oligosaccharide. The authors indicated that Fernie et al. [30] and Gruber and Levine [31] reported on a 45 KDa glycoprotein, endoglycosidase H-sensitive (containing high-mannose oligosaccharide), that is ''chased'' after 30 min into an 84–90 KDa mature, endoglycosidase H-resistant form of the G protein. The 45 KDa protein is not processed to mG protein in monensin-treated cells. The 45 KDa precursor and the G protein are produced in tunicamycin-treated cells. Lambert [32] studied the contribution of *N*- and *O*-linked oligosaccharides to the structural and functional make-up of the G glycoprotein and reported that oligosaccharides on the G glycoprotein are accessible to enzymatic removal.

Langedijk et al. [33] presented a model for RSV G glycoprotein, proposing that the polypeptide chain contains several independent folding regions, with the ectodomain consisting of a conserved central hydrophobic region located between two polymeric mucin-like regions. The central conserved region contains four conserved cysteine residues which can form disulfide bridges. The authors presented a three-dimensional (3D) model of the central region of RSV-G based on proteolytic digestion analysis.

Ghildyal et al. [34] reported that mG N-terminal residues 2–6 bind to the viral matrix (M) protein that is present at the cellular membrane for insertion.

Garcia-Beato and Melero [35] reported that the G protein C-terminal is partially resistant to protease digestion and that the G protein is glycosylated in a cell-dependent manner, influencing the antigenic epitopes in that part of the protein.

The functional properties of RSV sG glycoprotein

Teng et al. [36] isolated RSV mutants that were deleted in the G gene (ΔG) or in the sG (ΔsG). Both mutants replicated to a titer of $10^{6.3}$ log pfu/ml in Vero cells, but ΔG did not replicate in HEp-2 cells, while sG-deleted virus replicated in HEP-2 cells to the same titer as in Vero cells. The authors reported that since RSV G protein has a single binding site (aa184–198) for heparin, the authors constructed an RSV mutant with a deletion of aa187–197 in the G glycoprotein (GΔ187–197). This mutant infected HEp-2 cells and replicated as the wild-type virus. The ΔG RSV was found to be highly attenuated in mice, with no detectable virus in the upper respiratory tract and a lower respiratory tract infection in 60% of the mice. Replication of the sG virus (which expressed the sG and not the mG) was only moderately reduced in the upper and lower respiratory tract.

Johnson and Graham [37] reported that RSV sG glycoprotein induced the IL-5, IL-13 cytokines and eosinophilia by an IL-4-independent mechanism. The authors indicated that immunization of mice with sG glycoprotein or formalin-inactivated (FI-RSV) alum-precipitated RSV predisposed mice to the Th2

cytokine phenotype, which resulted in a more severe illness and pathology, decreased viral clearance and increased pulmonary eosinophilia upon subsequent RSV challenge. These immune responses are IL-4-dependent. The same increase in Th2 cytokines was achieved by infection with vaccinia virus expressing the RSV sG and mG glycoproteins. However, upon RSV challenge the sG-primed mice produced significantly higher levels of IL-5 and IL-13 mRNA and proteins than the mG-primed challenged mice. Priming of IL-4-deficient mice with sG demonstrated airway eosinophilia that was not dependent on IL-4. In contrast, airway eosinophilia induced by FI-RSV priming was dependent on IL-4 enhancement since significantly reduced eosinophilia was found in IL-4-deficient mice. The reason for the increased eosinophilia will be discussed.

Identification of amino acid domains in RSV G glycoprotein involved in the induction of the skewing of the Th1/Th2 cytokine balance in favor of Th2 cytokines

Elliott et al. [38] constructed two new RSV recombinants by reverse genetics: (1) deleting aa151 to 221 from the central ectodomain of the mG glycoprotein, and (2) a recombinant (rRSV) with a deletion of aa178 to 219 in the mG glycoprotein. This approach was based on the finding that a peptide spanning aa149–200 of the mG glycoprotein primed several strains of naïve inbred mice for a polarized Th2 cytokine response. Infection of BALB/c mice primed with G glycoprotein with the two rRSV deletion mutants did not induce pulmonary eosinophilia, suggesting that the deleted aa domain contains a sequence that is involved in the induction of eosinophilia in mice.

Maher et al. [39] generated replication-competent rRSV lacking secreted G glycoprotein or just the mG glycoprotein. The recombinant replicated well in HEp-2 cells, but after primary intranasal infection in mice the mG virus replication was reduced 10-fold compared to parental RSV. No apparent disease or pulmonary cellular infiltration was observed. Mice infected with the mG-deleted RSV developed antibody response and were fully protected against RSV challenge with the parental virus. The authors concluded that ''engineered RSV mutants lacking the ability to secrete the sG protein are thus promising vaccine candidates''.

Schwarze and Schauer [40] used a spontaneous variant of human RSV (RSV-ΔsG) with a mutated second start codon of the G protein gene preventing transcription of secreted G protein. It was reported that this mutant replicated in a human alveolar epithelial cell line, and induced increased production of proinflammatory chemokines and activation of the transcription factor NFκB. In mice infected with RSV-ΔsG, viral titers were increased 50-fold compared to wild type. Influx of eosinophils and macrophages to the lung and the levels of IFN-γ and IL-10 in the BAL fluid were significantly increased.

Arnold et al. [41] analyzed the inflammatory response of human lung epithelial cells (A549) infected either with RSV-WT or RSV-ΔsG and reported that at 20 h p.i. RSV-ΔsG *in vitro* induced increased cell surface expression of ICAM-1 on A549 cells and enhanced release of the chemokines IL-8 and RANTES (compared to RSV-WT). According to the authors, ''the data suggest that RSV reduces by the production of sG protein the detrimental inflammatory response to support its own replication''. The chemokine RANTES is a member of the C–C chemokine family and is a chemotactic and activation factor for monocytes, basophils and eosinophils, but not for neutrophils. The enhanced release of RANTES from RSV-ΔsG-infected A549 cells during the first 24 h p.i. suggested that the increase in the level of the C-X-C chemokine IL-8 released from lung epithelial cells, a chemotactic factor and activator for neutrophils, may be responsible for permanent accumulation of polymorphonuclear cells in the lungs.

Braciale [42] reviewed the effect of vaccination of mice with RSV G protein on the generation of $CD4^+$ T cells and memory $CD8^+$ T cells to G protein and found suppression of the development of a memory $CD4^+$ Th2 effector response after challenge infection.

Polack et al. [43] studied the function of the conserved region in the ectodomain of RSV G glycoprotein which includes a 13-aa segment (aa164–176) that is conserved in RSV strains A and B and in all wild isolates of RSV, and overlaps four cysteine residues (aa positions 173, 176, 182, 186) held by disulfide bonds between aa173–186 and aa176–182 [44]. The authors incubated purified monocytes with purified RSV F or G glycoproteins, or a combination of F plus G glycoproteins, and examined supernatant fluid for IL-6 production. Incubation of monocytes with F glycoprotein elicited high levels of IL-6, while the G glycoprotein did not induce IL-6 production by monocytes. Monocytes incubated with both F and G glycoproteins induced a 1.5 log decrease of IL-6 production. A similar effect was noted when IL-1β or IL-10 was added to F glycoprotein-treated monocytes. To determine if the conserved central region of G glycoprotein is responsible for the inhibition of the inflammatory cytokine, purified monocytes were incubated with F protein in combination with increasing concentrations of a synthetic

peptide representing aa164–189 of G glycoprotein of group A, which includes the 13-aa segment of the cysteine-rich region (GCRR). It was reported that increasing concentrations of the peptide led to a dose-dependent inhibition of F-mediated IL-6 production. Another peptide (aa273–288) within the mucin-like domain of the G glycoprotein did not affect F-induced IL-6 production. The authors studied whether RSV G glycoprotein inhibited cytokine production during RSV infection of monocytes. A wild type virus and a live recombinant with a deletion in the G gene (ΔG) were used and the supernatant fluids at 18 h p.i. were tested for cytokines. It was found that RSV-ΔG virus infection led to increased production of IL-6 and IL-1β in a dose-dependent manner compared to RSV wild-type infection. Using an rRSV that expressed the mG glycoprotein caused increased IL-6 production, while UV-inactivated RSV-ΔG infection led to enhanced cytokine production, demonstrating that a live virus is not necessary for G glycoprotein modulation. To elucidate the role of GCRR (GΔ172–187), purified human monocytes were incubated with rRSV lacking the GCRR, and IL-6 levels were reported to be higher than after RSV infection. It was also demonstrated that the fractalkine (FKN) motif in the G glycoprotein did not play a role in the modulatory effect on cytokine production.

A functional domain in RSV sG glycoprotein that causes eosinophilia and weight loss

Sparer et al. [45] used RSV mutant viruses containing frameshifts that altered the carboxy terminus of sG glycoprotein to dissect the region of G protein responsible for enhanced disease and the region involved in protection against RSV challenge. In addition, the authors used recombinant vaccinia virus (rVV) expressing RSV G. Mice were sensitized by scarification with rVV expressing the entire viral G glycoprotein or specific G peptides. Amino acid residues 193–205 were identified as being responsible for G glycoprotein-induced weight loss and lung eosinophilia. Sensitization of mice with rVV expressing the peptide aa124–203 induced lung eosinophilia and weight loss, similar to the disease in the mice that was caused by vaccination with FI-RSV vaccine. The authors suggested that alterations of residues 193–203 abolished a strong $CD4^+$ T cell response.

Tebbey et al. [46] reported that *in vitro* stimulation of spleen cells from natural G protein-primed mice showed dominant proliferative and cytokine (IFN-γ and IL-5) responses to an RSV peptide encompassing aa184–198. Mice vaccinated with the aa184–198 G peptide conjugated to keyhole limpet hemocyanin showed significant pulmonary eosinophilia after challenge with live RSV. Immunization with the aa208–222 G peptide did not cause pulmonary eosinophilia after RSV challenge. In vivo depletion of mouse $CD4^+$ T cells abrogated the effect of the G peptide. According to the authors, ''the data showed that the G peptide aa184–198 (AICKRIPNKKPGKKT) primed the pulmonary eosinophilia by stimulating the expansion of $CD4^+$ T cells destined to secrete IL-5''.

Srikiatkhachorn et al. [47] analyzed the cytokine profile of effector $CD4^+$ T cells elicited by RSV G glycoprotein in a murine model of RSV infection. Groups of mice were primed either with vaccinia virus recombinant expressing RSV G glycoprotein (rVV-G) or control vaccinia virus. The mice were challenged with live RSV 3 weeks following priming and a portion of the lungs was removed for histology and morphometric analysis 5 days later. The remaining lung tissue was used for isolation of infiltrating mononuclear cells. It was reported that the RSV-G-sensitized mice had marked lung eosinophilia, and the levels of IL-4 and IL-5 produced by the infiltrating mononuclear cells correlated with the degree of eosinophilia. To define the potential epitope(s) contained in RSV-G, overlapping 15 aa peptides spanning the entire RSV-G were tested for their ability to stimulate cytokine release from heterogenous populations of RSV G-specific T cells from individual RSV-infected rVV-G-primed mice. The authors identified only one synthetic peptide that was recognized by these bulk cultures of RSV-G-specific $CD4^+$ T effector cells. The peptide was defined as an epitope spanning aa183–197 of G glycoprotein of RSV A2 strain. It was also reported that the aa183–190 G peptide induced lung mononuclear cell populations from individual RSV-G-sensitized mice to produce Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-5). These cells did not respond to RSV-G peptide aa1–15. The authors concluded that non-MHC genes are crucial for the development of pulmonary eosinophilia and the Th2 response in mice.

Johnson et al. [48] compared the amino acid sequence of RSV-G glycoprotein of three RSV strains: Long, A2 and 18537. The A2 strain synthetic peptide aa183–197 [47] (183WAICKRIPNKKPGKK197) is conserved in the three RSV strains.

Varga et al. [49] more precisely defined the $CD4^+$ T cell epitope aa183–197 domain of the RSV G protein, and created a panel of amino- and carboxy-terminal truncated and single alanine-substituted peptides spanning aa183–197. The mutated peptides were used

to examine *ex vivo* the cytokine response of memory effector $CD4^+$ T cells infiltrating the lungs of G-primed, RSV-infected mice using intracellular cytokine staining and/or ELISA of effector T cell culture supernatants. It was reported that a series of consecutive deletions from the N-terminus are stimulatory until the isoleucine at position aa185 is deleted. Consecutive C-terminus deletions of the synthetic peptides of different lengths spanning aa183–197 revealed that the loss of lysine (K) at position 192 ablated the ability of the peptide to stimulate IFN-γ production. It was found that alanine-substituted peptides at aa 185 (I), 186 (C), 187 (K), 188 (R) and 189 (I) were unable to induce IFN-γ, while aa 190 (P), 191 (N) stimulated IFN-γ to 50% of the unchanged peptide aa183–197 and aa 192 (K) and 193 (K) stimulated IFN-γ to 25 and 40%, respectively. The authors concluded that G-protein-specific memory cells in RSV-infected BALB/c mice are directed against a single I-E^d-restricted immunodominant epitope, and demonstrated that the majority of RSV G protein-specific $CD4^+$ T cells express a restricted Vβ14 T cell receptor.

The CX3C chemokine motif in RSV G glycoprotein

Tripp et al. [50] reported on the RSV G glycoprotein aa sequence containing a domain that mimics the CX3C domain characteristic of the chemokine fractalkine (FKN) (the only member of the CX3C chemokine group gamma). Both glycoprotein RSV G and FKN have heparin-binding domains (HBDs) which interact with the glycosaminoglycans (GAGs) on cell membranes. The G glycoprotein contains a CX3C aa motif at aa182–186, but no other chemokine motif (C, C–C, CXC). Although FKN has minimal amino acid homology with G glycoprotein, the two G glycoproteins (289 or 299 aa) and FKN (397 aa) exist as membrane-bound glycoproteins and secreted soluble protein forms. In addition, within the conserved CX3C region of G (aa169–191) there is 42% homology with the FKN domain (aa33–55). The authors reported that RSV G glycoprotein binds to FKN receptor CX3CR1 and induces leukocyte chemotaxis and facilitates RSV infection of cells. Since both RSV G and FKN can bind to cells via HBD–GAG interactions, the authors included heparin in the binding experiments to allow the G glycoprotein to bind the CX3CR1 expressed by 293 cells. The authors reported that binding of RSV sG was minimally inhibited by FKN (20–34%), while addition of FKN to heparin almost completely inhibited G glycoprotein binding (78–84% to 85–98%). Peptide RT33 aa (181TCWAICKRIPNK192) of RSV-G, together with heparin, inhibited the binding of RSV G glycoprotein to cells. Uninfected Vero cells, of which 31–51% of the cells expressed CX3CR1, were used to study the effect of treatment with heparin, FKN, G glycoprotein, G peptides and anti-CX3CR1 antibodies. Treatment of Vero cells with heparin caused a 66% reduction of RSV plaques. Addition of G glycoprotein prior to RSV infection caused a 98% plaque reduction. Treatment with FKN, RT33 peptide, which contains the FKN motif, and anti-CX3CR1 antibodies caused 97%, 92% and 91% plaque reduction, respectively. RSV-G peptides that did not have the CX3C motif did not increase heparin-associated plaque reduction. The ability of RSV G glycoprotein to mimic the capability of FKN to induce leukocyte chemotaxis was studied in a modified Boyden chamber. It was reported that antibodies to CX3CR1 inhibited leukocyte chemotaxis toward either RSV-G glycoprotein or FKN. The viral glycoprotein G inhibited leukocyte chemotaxis toward FKN by approximately 37%. Similar results were observed in chemotactic studies with human PBMCs, of which 38–49% express CX3CR1; they inhibited leukocyte chemotaxis by 8–30% after incubation for 6 h at 37°C, possibly due to receptor endocytosis. RSV-G and FKN induce leukocyte chemotaxis by mechanisms that involve binding to the FKN receptor CX3CR1.

It should be noted that the RSV G peptide RT33 (aa181 TCWAIC KRIPNK 192) contains two functional domains: the CX3C motif and a T cell antigen domain that induces the release of large amounts of the Th2 cytokines IL-4 (inhibitor of Th1 cells and B cell inducer to IgE synthesis), IL-5 (the cause of eosinophilia), IL-10 and IL-13 from mast cells, basophils and monocytes. The cytokine IL-5 activates eosinophils and the G FKN domain directs them to the site of RSV infection.

The role of C–C and CXC chemokines in RSV infection

Tripp et al. [51], in a recent review, indicated that infants hospitalized with severe RSV disease have reduced IFN-γ levels in nasopharyngeal aspirates and reduced IFN-γ expression by PBMCs. These children are predisposed to asthma after a severe infection in early childhood, a disease characterized by an inflammatory process that is associated with increased levels of serum Th2-type expression of cytokines and chemokines by RSV-infected epithelial cells. The C–C chemokines attract lymphocytes and eosinophils to sites of inflammation and incite inflammatory responses by inducing chemokine release from eosinophils, basophils and mast cells. In infants and children infected by RSV, high levels of C–C chemokines were associated with severe bronchiolitis and inflammation. The C–C chemokines associated with RSV pathogenesis are

MIP-1alpha/CCL3 and RANTES/CCL5, which are found at high levels in aspirates of RSV-infected infants. The C–C chemokines interact with their specific receptors, CCR1 and CCR5, respectively, that are expressed on Th1 cells. The authors suggested that RSV-G or SH glycoproteins may impair Th1 responses mediated by these chemokines and CX3C chemokine FKN.

John et al. [52] used a murine model combining early RSV infection with cockroach allergen challenge, and examined the role of RSV-induced CCL5/RANTES production on the allergic airway response. The authors reported that RSV infection increased the levels of CCL5 mRNA and protein, peaking at days 8 and 12, respectively. In mice receiving RSV allergen challenge, the lungs collected at day 22 p.i. showed significantly decreased numbers of $CD4^+$ and $CD8^+$ T cells. Pretreatment with CCL5 antiserum resulted in decreased recruitment of inflammatory cells.

Tripp et al. [53] studied the kinetics of chemokine mRNA expression by pulmonary leukocytes following primary infection of 4–6-week-old female BALB/c mice infected with two RSV strains, one strain with G and SH genes (B1 group) and the other lacking these two genes (B2 group), and a third group of mice were infected with the JS strain of parainfluenza virus 3 (PIV-3). It was reported that the G and/or SH gene expression reduced the expression of MIP-1alpha, MCP-1 and IL-10 mRNA, chemokines that interact with receptors expressed by Th1 cells (CCR5, CXCR3 and CXCR5) and chemokine receptors preferentially expressed by Th2 cells (CCR3, CCR4 and CCR8). MIPs interact with CCR1 and CCR5, MCP1 interacts with the CCR2 receptor, and IL-10 interacts with the CXCR3 receptor on Th1 cells. The expression of MIP-1alpha, MCP-1 and IL-10 associated with G and/or SH glycoproteins impairs the Th1 response.

The CX3C chemokine fractalkine (FKN) in allergic asthma and rhinitis and the polarization of Th1 responses

Rimaniol et al. [54] studied the plasma FKN levels in 19 control subjects and 55 patients with symptomatic allergic rhinitis, asthma or both. The function of CX3CR1 was studied in circulating T-lymphocyte subpopulations. The authors reported that patients with symptomatic allergic rhinitis and asthmatic patients had increased circulating FKN levels, and CX3CR1 was up-regulated in circulating $CD4^+$ T lymphocytes. Twenty-four hours after allergen challenge, bronchoalveolar lavage fluid contained increased FKN concentration, and bronchial epithelial and endothelial cells expressed high levels of the membrane-bound FKN before and after challenge. Fractalkine is expressed by endothelial cells, epithelial cells, dermal cells, dendritic cells and neurons.

Fracticelli et al. [55] examined the role of FKN in polarized Th1 and Th2 cell responses. Proinflammatory signals, including LPS, IL-1, TNF-α and CD40 ligand (CD40L), induced FKN as did IFN-γ, which had synergistic activity with TNF-α. The Th2 cytokines IL-4 and IL-13 did not stimulate FKN expression and markedly reduced induction by TNF-α and IFN-γ. TNF-α alone or combined with IFN-γ also induced release of soluble FKN, which was inhibited by IL-4 and IL-13. The authors analyzed the interaction of FKN with natural killer (NK) cells and polarized T-cell populations. NK cells express CX3CR1. The FKN receptor was preferentially expressed by Th1 cells which respond to FKN, while Th2 cells do not respond to FKN. Endothelial cells in *Mycobacterium tuberculosis* granulomatous lymphadenitis showed immunoreactivity to FKN.

During the evolution of RSV to become a human pathogen it is possible that part of the FKN mRNA (as DNA) motif was inserted, into the RSV G gene near the sequence which codes for the T cell antigen. With the FKN motif the viral sG glycoprotein directs IL-5-activated eosinophils to the lungs.

RSV fusion (F) glycoprotein mediates immunity by TLR4 and CD14 expressed by innate immune cells

RSV F protein, mediator of the innate immune response

Kurt-Jones et al. [56] examined the interactions of several RSV proteins with human monocytes and demonstrated that RSV F glycoprotein induces proinflammatory cytokines, and this response is dependent on the cellular expression of CD14 and TLR4. The latter are essential for initiating the innate response to lipopolysaccharide (LPS) from Gram-negative bacteria, mycoplasmas, spirochetes and fungi. The authors studied the role of CD14 in the response to RSV F protein using $CD14^{-/-}$ knockout mice. Macrophages from $CD14^{+/-}$ homozygous mice produced IL-6 and IL-1β after stimulation with RSV F protein. Macrophages from the $CD14^{-/-}$ mice did not produce these cytokines after RSV F stimulation. It was also reported that RSV replicated in TLR4-deficient mice to a higher titer than the control mice.

Polack et al. [43] reported that RSV F protein interacts with the CD14/TLR4 complex on monocytes

and stimulates production of proinflammatory cytokines IL-6, IL-1β and IL-8 by promoting nuclear translocation of the nuclear transcription factor NF-κB, which plays important role in neutrophil and macrophage chemotaxis and activation during RSV infection. In addition, F protein elicits the synthesis of neutralizing antibodies, has CTL epitopes in human and mice and induces increased production of Th1 cytokines, IL-2, IL-12 and IFN-γ.

Structural properties of RSV fusion (F) protein core

Zhao et al. [57] used a computer program to predict the conformation of the RSV fusion (F) protein core and described the two heptad-repeat regions of the protein. These regions form trimer-of-hairpin-like structures, similar to fusion proteins of several enveloped viruses. The hairpin structure brings the viral and cellular membranes into close apposition, facilitating membrane fusion and subsequent entry of the virion protein core into the cell cytoplasm. The authors showed that peptides corresponding to the heptad-repeat regions from the N-terminal (HR-N) and the C-terminal (HR-C) segments of the RSV F protein form a stable α-helical trimer of heterodimers. The RSV N/C complex was crystallized and its X-ray structure was determined at 2.3 Å resolution. The complex is a six-helix bundle in which HR-N polypeptides form a three-stranded, central coiled coil, and HR-C polypeptides pack in an anti-parallel manner into hydrophobic grooves on the coiled-coil surface. The authors concluded that RSV F protein has a remarkable structural similarity to the fusion core of other viruses including HIV-1 gp41 fusion protein.

Synthesis of RSV fusion protein in infected cells

Bolt et al. [58] reported that RSV F protein is synthesized as a non-fusogenic precursor protein (F_o), and that during migration to the cell surface the F_o protein is cleaved into disulfide-linked F_1 and F_2 subunits by the proteolytic enzyme furin.

Rixon et al. [59] studied the synthesis of RSV F glycoprotein in infected Vero cells and found the presence of a single F1 subunit and at least two different forms of F2 subunits, F2a (21 KDa) and F2b (16 KDa) (F2 a/b). Enzymatic deglycosylation of F2a/b produced a single 10 KDa polypeptide. The detection of F2a/b was dependent upon post-translational cleavage by furin. F1 subunit interacted with F2a/b via disulfide bonding to produce equivalent F protein trimers.

RSV F glycoprotein-specific CD8$^+$ T cells during experimental virus infection

Chang et al. [60] screened a panel of overlapping synthetic peptides corresponding to the RSV F protein to identify F-specific T cells in the lungs of RSV-infected mice by flow cytometry. A dominant H2K^d-restricted epitope ($F_{85–93}$ aa KYKNAVTEL) was identified by CD8$^+$ T cells from BALB/c mice and enumerated the F-specific T cell response in the lungs of RSV-infected mice. It was reported that during primary infection $F_{85–93}$-specific effector CD8$^+$ T cells constituted approximately 4.8% of pulmonary CD8$^+$ T cells at the peak of the primary response (day 8), whereas CD8$^+$ T cells to matrix 2 protein constituted approximately 50% of the CD8$^+$ T cell population in the lungs. When RSV F-immune mice undergo an RSV challenge, the F-specific CD8$^+$ T cell response is accelerated and dominates, whereas the primary response to matrix 2 epitope in the lung is reduced approximately 20-fold. The authors also reported that the effector CD8$^+$ T cells isolated from the RSV-challenged mice exhibited lower IFN-γ synthesis and significantly impaired cytolytic activity *ex vivo*.

It is possible that the increased levels of Th2 cytokine production in RSV-infected mice, especially the increased IL-4 level released from FcϵRI$^+$ hematopoietic cells, inhibit Th1 cell cytokine synthesis and the activation of CTL precursor cells to become antiviral CTLs.

CTL precursor (CTLp) frequencies in BALB/c mice after acute RSV infection

Tripp and Anderson [61] endeavored to understand the immune response of BALB/c mice to RSV infection and to FI-RSV vaccine. CTLp frequencies were determined in bronchoalveolar lavage (BAL) samples and spleen lymphocytes from BALB/c mice that were intranasally infected with live RSV or intramuscularly inoculated with FI-RSV. The authors reported that both class I- and II-restricted CTLps were detected by day 4 or 5 p.i., peaking at day 7 p.i. For spleen cells, MHC class I- and II-restricted CTLps to live RSV were similarly detected. However, the class II-restricted CTLp frequencies in BAL following RSV infection were less than class I-restricted CTLp frequencies through day 4 p.i., during which class I-restricted CTLp frequencies remained elevated and declined by 48 days p.i. The frequencies of class II-restricted CTLps in the BAL were 2- to 10-fold less than class I-restricted CTLps. In contrast, class II-restricted CTLps predominated in FI-RSV vaccinated mice. RSV challenge of

vaccinated mice increased the frequency of class I-restricted CTLps at day 3 p.i. but did not enhance class II-restricted CTLp frequencies. The authors speculated that ''the shift in CTLp frequency from predominant class II-restricted response following FI-RSV vaccination to a predominantly class I-restricted response following live RSV challenge is related to the shift from Th2 type cytokines toward Th1-type cytokines after RSV infection in FI-RSV-immunized mice''.

Srikiatkhachorn and Braciale [62] used mice that were primed with vaccinia virus recombinants expressing RSV-G (VVG) or RSV-F (VVF) to study the mechanisms underlying the differences between RSV-G, which induces a Th2 response and RSV-F which induces a Th1 response. BALB/c mice primed with RSV-F mounted a strong RSV-specific, MHC class I-restricted cytolytic response. Using RSV-M2 (matrix) protein strong $CD8^+$ T cell epitope, inserted into vaccinia virus harboring RSV-G, resulted in suppression of eosinophil recruitment into the lungs of treated mice upon subsequent challenge with RSV. This finding was supported by results in $CD8^+$ cells from β2-microglobulin-deficient KO mice, in which priming with RSV-F resulted in the development of a marked pulmonary eosinophilia.

RSV non-structural proteins NS1 and NS2 inhibit the induction of the cellular type I interferon synthesis by the RSV-infected cells

Cells infected by RNA negative-strand viruses respond to infection by activation of the human MxA gene, a 76 KDa protein with GIPase activity which is induced to a high level and is present in the cytoplasm of type I IFNα,β-treated human cells. Atreya and Kulkarni [63] compared the antiviral effects of human type I IFN and IFN inducers poly (I:C) on the replication of RSV A2 in human lung epithelial cells (A549 cell line) or fibroblasts (MRC-5). The authors tested whether MxA protein was induced in these cells in response to type I IFN and reported that RSV A2 possessed replicative strategies that are resistant to the antiviral effects of type I IFNα and MxA.

Teng and Collins [64] used a reverse genetics system for recovering RSV recombinants (rRSV) from cDNA. This system involves the intracellular co-expression of the N, P, L and M2–1 proteins and the RSV antigenome, under the control of the T7 promoter, in cells concomitantly infected with vaccinia virus recombinant expressing T7 RNA polymerase. The authors ablated expression of the NS2 gene by introducing termination codons or excising the complete gene from the antigenome cDNA clone, and reported that virus revertants were not recovered from the recombinant ΔNS2 RSV that formed very small plaques. The authors concluded that although the exact function of RSV NS2 protein remains unclear, this protein is not essential for virus replication, and they suggested that the ΔNS2 RSV is a candidate for vaccine development.

Schlender et al. [65] studied the function of bovine RSV (BRSV) NS1 and NS2 genes by creating deletion of NS1 and deletions of NS1 and NS2 in BRSV mutants. It was reported that the deletion mutant replication in MDBK cells resulted in a 5000- to 10,000-fold reduction of virus titers. It was found that supernatant from BRSV-infected MDBK cells restrained the growth of NS1/2 deletion mutants in Vero cells, and the factor responsible was identified as type I IFN by neutralization of the inhibitory effect with anti-IFN-α antibodies. All NS deletion mutants were equally repressed, indicating an obligatory cooperation of NS1 and NS2 in antagonizing IFN-mediated antiviral mechanism.

Young et al. [66] studied the mechanism by which paramyxovirus non-structural genes inhibit the induction of interferon-stimulated genes (ISGs) in paramyxovirus-infected cells. Type I IFNs (IFN-α and IFN-β) are directly induced in target cells by viral infection, while type II IFN (IFN-γ) is directly produced in response to viral infection by Th1 cells and NK cells. Both IFN types I and II bind to independent cell surface receptors and activate distinct but related signal transduction pathways, leading to the activation of an overlapping set of ISGs. The binding of type I IFNs to their cognate receptors induces the receptor-associated tyrosine kinases Jak1 and Tyk2 to phosphorylate the transcription factors STAT1 and STAT2. When the phosphorylated STAT1 and STAT2 heterodimerize and translocate into the nucleus, they associate with the IRF-family transcription factor p48, forming the IFN-stimulated gene factor 3 (ISGF3) which activates the transcription of ISREs within their promoters. Following the binding of IFN-γ to type II IFN receptor, the receptor-associated tyrosine kinases Jak1 and Jak2 phosphorylate STAT1, which homodimerizes to form g-activated factor (GAF) that binds to the g-activated sequence (GAS). Phosphorylation of amino acid S727 is essential for the full transcriptional activity of GAF. The authors determined how paramyxoviridae members SV5, SEV, RSV, hPIV2 and hPIV3 circumvent the IFN response of infected cells and concluded that ''paramyxoviridae members achieved the interference with IFN signaling by different molecular mechanism''.

Bossert et al. [67] studied the mechanism of BRSV NS1 and NS2 inhibition of the induction of IFN-β

stimulated genes (ISGs). Infection of cells with BRSV ΔNS1/2 resulted in a significant increase in the cellular IFN-β gene promoter activity. This induction of IFN-β promoter depends on the activation of three transcription factors, NF-κB, ATF-2/c-Jun and IFN regulatory factor 3 (IRF-3). Phosphorylation of IRF-3 was observed only after infection with BRSV ΔNS1/2 recombinant. Wild-type BRSV NS protein-mediated inhibition of IRF-3 activation that has considerable impact on the pathogenesis and immunogenicity of BRSV.

Ramaswamy et al. [68] studied the selective viral effects of RSV infection on type I IFN-dependent signaling pathway and reported that type I IFN-induced activation of transcription factor STAT1 in RSV-infected cells was impaired, while type II IFN activation was not affected. RSV infection of airway epithelial cells resulted in decreased STAT2 expression, revealing the molecular mechanism for viral inhibition of type I IFN JAK-STAT pathway, since non-specific pharmacologic inhibition of proteasome function in RSV-infected cells restored STAT2 activity and IFN-dependent activation of STAT1. RSV infection of epithelial cells in the airways modulated the type I IFN JAK-STAT pathway, mediated by proteasome-dependent degradation of STAT2. The decreased antiviral gene expression in RSV-infected airway epithelial cells allows RSV replication and the establishment of a productive viral infection through subversion of IFN-dependent immunity since airway levels of type I IFN, commonly induced by RSV, could inhibit viral replication. Joshi et al. [69] speculated that ''RSV evolution did not generate mechanisms to inhibit type II IFN signaling because IFN-γ levels in airways are often below the level required to inhibit viral gene expression during infection''.

Spann et al. [70] reported that human wild type RSV is a poor inducer of IFN-α/β, while the NS1 and NS2 deletion mutant hRSVΔNS1/2 induced high levels of type I IFN-γ in human pulmonary epithelial (A549) cells and macrophages derived from human peripheral blood monocytes. The authors investigated the effect of hRSV infection and of the ΔNS1 and/or NS2 on the expression of the newly described antiviral cytokine IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) and their receptor IL-28R [71, 72]. These IFNs are broadly expressed, are inducible by double-stranded RNA (dsRNA) or virus infection, bind to a heterodimeric receptor, activate JAK/STAT signal transduction pathway, up-regulate genes that render the cell resistant to infection. IFN-λ appears to be an IFNα/β-like cytokine with pleiotropic effects that include potent antiviral activities. The authors examined the expression of IFN-λ1 and IFN-λ2/3 and reported that RSV ΔNS1/2 induced an increase of IFN-λ1 and IFN-2/3, peaking at 18 h p.i. RSV ΔNS1 displayed modestly elevated levels of IFN-γ, peaking at 26 h p.i.

The properties of RSV small hydrophobic (SH) glycoprotein

RSV SH glycoprotein gene codes for a protein that contains 65, 64 or 81 amino acids

Alansari and Potgieter [73] reported that the SH glycoprotein is anchored in the cellular membrane by the N-terminus so that the C terminal amino acids are extracellular. The RSV SH gene has two initiation codons, with the second initiation codon, at nucleotide 23, generating truncated SH glycoprotein molecules. Alignment of SH glycopeptide aa sequence with that of RSV G glycoprotein revealed neither a fractalkine sequence nor the B cell antigenic epitope. The biological function of SH glycoprotein biological activity was not reported.

Wilson et al. [74] studied the small hydrophobic (SH) gene, which encodes a type I membrane protein of 57 aa residues, and explored the function of the SH protein encoded by mumps virus (MuV) and simian virus 5 (SV5), members of the Paramyxoviridae family. The authors reported that both SV5 and MuV ectopically expressed SH blocked activation of NFκB by TNF-α signaling.

Attempts to develop a safe immunogenic and protective anti-RSV vaccine

The life-threatening RSV infections in infants during their first year of life, and the danger of acute RSV infections of the upper and lower respiratory tracts of elderly people [75, 76] require the development of a safe and protective vaccine. Nevertheless, while during the second half of the twentieth century protective antiviral vaccines were developed against most childhood viral diseases, eliminating them from the developed nations, an anti-RSV vaccine was not developed. Kim et al. [77] analyzed the blood of FI-RSV-immunized children and reported the presence of high titers of non-neutralizing serum antibodies, presumably anti-RSV IgE. Histological examination of lung tissue from one of the dead infants revealed eosinophilia. The connection between the presence of non-neutralizing antibodies and lung eosinophilia was not known thirty years ago.

RSV sG glycoprotein causes transient allergy in infected patients

Hancock et al. [78] examined the feasibility of using the highly purified RSV G glycoprotein as a subunit vaccine against RSV in a murine (BALB/c) model, with or without the viral F glycoprotein, with the potent adjuvant QS-21. The authors reported that immunization of mice with RSV G/QS-21 vaccine generated immune responses characterized by a low level of antigen-dependent NK cell activity, elevated levels of IL-5 and eosinophilia in the bronchoalveolar lavage fluid after RSV challenge. Splenic immunocytes secreted IL-5, not IFN-γ, after *in vitro* stimulation with purified whole virus antigens. The pulmonary eosinophilia was similar to that induced by the FI-RSV vaccine in the previous clinical trials. The F/QS-21 vaccine elicited more Th1-type response, but no eosinophilia nor elevated levels of IL-5, and splenic immunocytes secreted large quantities of IFN-γ.

Johnson et al. [79] vaccinated BALB/c mice with recombinant vaccinia virus which expressed the RSV sG (VV48) and found that the vaccinated mice responded with a more severe illness following RSV challenge than did mice primed with vaccinia virus or vaccinia virus expressing the membrane-anchored G glycoprotein (VV48I). The authors concluded that priming with RSV sG glycoprotein promoted eosinophil recruitment associated with IL-5 production, and that illness is associated with the movement of eosinophils from the interstitital compartment in the lung to the alveoli. Johnson et al. [80] constructed recombinant vaccinia viruses co-expressing mouse IL-4 and IFN-γ. The expression of IL-4 increased the replication of the virus vector, while expression of IFN-γ reduced vaccinia virus replication. Mice immunized with vaccinia virus expressing RSV G glycoprotein and IFN-γ were protected, even though replication of the vector was diminished. The study led to the concludion that ''the co-expression of cytokines and other immunomodulators has the potential to improve the safety of vaccinia vectors while improving the immunogenicity of vaccine antigens''. In a recent study, Johnson et al. [81] evaluated the role of RSV G and its immunodominant region in induction of an aberrant immune response during FI-RSV immunization. BALB/c mice were immunized with FI preparations of (i) w.t. RSV or a recombinant RSV containing a deletion of the entire G gene, (ii) the G gene, with a deletion of the nucleotide sequence coding for aa187–197, or (iii) deletion of the SH gene. The authors reported that deletion of the G glycoprotein or its epitope did not reduce illness, cytokine production or eosinophilia as compared to immunization with FI-RSV. The authors concluded that ''RSV G should not be excluded from potential vaccine strategies''.

Synthetic peptides designed according to RSV G protein epitopes as vaccines

Huang and Anderson [82] used an *E. coli*-grown vector encoding a fragment of thioredoxin (Trx) fused to a central RSV (Long strain) nucleotide sequence coding for the G protein aa128–229. Site-directed mutagenesis of aa185–193 was employed to determine the importance of this epitope for the induction of eosinophilia in immunized mice that were subsequently challenged with RSV. It was reported that mutations of C186A, K187A, R188A, P190A and N191A in Trx-G polypeptide reduced the number of eosinophils recovered in the bronchoalveolar lavage. However, Th2 cytokine mRNA levels in the lung were dramatically elevated following infection with w.t. RSV. The strongest response was seen with IL-13, along with IL-10, IL-4 and IL-5. RSV-challenged mice which had been immunized with the G peptide I189A, P190A, U192A or K193A mutants had high levels of eosinophils. In contrast, the K187A and R188A G peptide mutants were poor inducers of IL-13 and IL-10 and eosinophilia despite being good inducers of IL-4. The authors concluded that ''the search for a safe and effective vaccine remains elusive due to an unknown number of beneficial as well as immunological determinants on both G and other constituents''.

RSV vaccines tested in monkeys

Yusibov et al. [83] engineered a 21-mer peptide representing aa170–190 of RSV G protein fused with the alfalfa mosaic virus, (AlMV) coat protein, producing recombinant A1MV particles containing the RSV-G peptide (VMR-RSV) on the surface of virions. Immunogenicity was tested *in vitro* on human dendritic cells and in vivo in non-human primates. The human dendritic cells interacted with the VMR-RSV peptide and generated vigorous $CD4^+$ and $CD8^+$ T cell responses. Non-human primates (two rhesus macaques and two cynomolgous macaques) that were vaccinated with VMR-RSV particles responded by mounting strong cellular and humoral anti-RSV immune responses. According to the authors, these ''studies clearly demonstrate that plant virus particles generated strong T cell responses in human DCs and both T and B cell responses to the RSV peptide in the non-human monkeys. The data support the potential use of this platform for delivery of component vaccines for RSV''.

DeSwart et al. [84] reproduced the FI-RSV vaccine [77] and vaccinated cynomolgous macaques. The vaccine induced specific virus-neutralizing antibody responses in the monkeys that were accompanied by strong lymphoproliferative responses. The vaccine induced RSV-specific T cells to produce predominantly IL-13 and IL-5 and intratracheal challenge with macaque-adapted RSV at 3 months after the third vaccination elicited a hypersensitivity response associated with lung eosinophilia and a rapid boosting of IL-13-producing T cells in the vaccinated animals, but not in the FI-measles virus-vaccinated control animals. Two of seven FI-RSV vaccinated animals died 12 days after RSV challenge after developing the strongest lymphoproliferative responses associated with the most pronounced Th2 cytokine phenotype within their group. The authors ''hypothesized that an IL-13-associated asthma-like mechanism resulted in airway hyperreactivity in these animals''.

Whitehead et al. [85] administered wild-type RSV (strain A2) recombinants, from which NS2 and SH genes had been separately deleted, to chimpanzees to evaluate the levels of attenuation and immunogenicity of these deletion mutants. The rRSV-A2ΔNS2 replicated to a moderate level in the upper respiratory tract, was highly attenuated in the lower respiratory tract, and induced significant resistance to challenge with w.t. RSV. The authors reported on an RSV ΔSH deletion mutant that was incorporated into a recombinant form of the cold-passaged, temperature-sensitive candidate vaccine, *cpts*248/404, and was evaluated for safety in seronegative chimpanzees as a possible vaccine for humans.

Schmidt et al. [86] used reverse genetics to develop a two-component, trivalent live attenuated vaccine against human parainfluenza virus type 3 (HPIV3) and RSV subgroups A and B. The backbone of each of the two components of this vaccine was the attenuated recombinant bovine/human PIV3 (rB/HPIV3) in which bovine SH and F protective antigens were replaced by their HPIV3 counterpart. This virus chimera retained the well-characterized host range attenuation phenotype of BPIV3, which appears to be appropriate for immunization of young infants. The authors inserted the G and F genes of RSV into rB/HPIV3 and this recombinant, as well as the parental strain, replicated in the respiratory tract of rhesus monkeys and induced a robust immune response to both RSV and HPIV3. Murphy and Collins [87] reported on the use of reverse genetics systems based on RSV A2, PIV3 strain JS and bovine PIV3 to develop a live attenuated RSV vaccine. Fan and Mei [88] used the disulfide bond isomerase (DsGA) of *E. coli* as an effective carrier protein to increase the longevity of CTL response *in vivo*, and constructed vectors which co-immunized with chimeric CTL epitope of G protein fragments (aa128–229, 130–230, 128–229 and chimeric CTL epitope M2: aa81–95). It was reported that the peptide constructs enhanced protein- and virus-specific CTL responses. In addition, high levels of antibodies to RSV and neutralizing antibodies were induced.

Harcourt et al. [89] indicated that current vaccine strategies that include live attenuated virus, protein subunit, and DNA vaccines were not sufficiently safe. Therefore, the authors examined CD40 ligand (CD40L) as an immune modulator to enhance durability of DNA vaccines encoding RSV F and G glycoprotein in BALB/c mice. The addition of CD40L to RSV DNA vaccines encoding the F protein enhanced virus clearance.

Bartholdy et al. [90] generated a $CD8^+$ T memory cell response to RSV using a DNA vaccine construct encoding the dominant K^d-restricted epitope from the viral transcription terminator protein M2 (aa82–90), covalently linked to human β2-microglobulin. The DNA construct was injected to BALB/c mice by cutaneous gene-gun immunization. Intranasal RSV challenge accelerated $CD8^+$ T cell responses in pulmonary lymph nodes and virus clearance from the lungs was enhanced.

Vaccine containing attenuated recombinant RSV ΔG and ΔSH glycoproteins

Karron et al. [91] studied a cold-passaged (cp) RSV B candidate vaccine (designated B1cp-52/2B5 (cp-52)) that was derived by passage of RSV B1 w.t. virus 52 times at low temperature (21–32°C). This virus was restricted in replication in vivo, but induced RSV neutralizing antibody response in cotton rats, African green monkeys and chimpanzees. The authors described the phase I evaluation of cp-52 candidate vaccine in adults, children and infants. This attenuated virus has a large deletion that ablated the SH and the G glycoproteins, and was poorly infectious and overattenuated in humans, but replication-competent *in vitro*.

The authors concluded that although the cp-52 mutant virus is not an appropriate RSV B vaccine candidate for RSV seronegative children, and concluded that ''the use of cDNA technology will allow the construction of a series of different recombinant viruses''.

Kahn et al. [92] used an attenuated, non-propagating vesicular stomatitis virus (VSV) deleted of the VSV G gene (VSVΔG) and incorporated the RSV G gene or

F gene into the VSV genome. Intranasal vaccination of mice with the VSV–RSV recombinant expressing RSV G or RSV F elicited RSV-specific antibodies in the serum, as well as anti-RSV neutralizing antibodies. However, the mice were not protected against RSV challenge.

Corvaia et al. [93] constructed a prokaryotic vector expressing a recombinant RSV G subunit protein containing aa130–230 (G2Na) and albumin-binding domain (BB) of *Streptococcus* G protein and studied the safety of this protein (BBG2Na) in a mouse model. The aim of the study was to characterize the cytokine pattern of mice immunized with BBG2Na and to evaluate the influence of an RSV challenge by studying the Th2 cytokine response and serum IgE level. The authors immunized BALB/c mice with the BBG2Na vaccine prepared in alhydrogel adjuvant and reported that IgE antibodies to. G2Na and IL-5 were found in the serum. Isolated splenocytes that were stimulated with the vaccine released large amounts of IL-5 and some IL-4. However, RSV challenge of the immunized mice by the intranasal route did not lead to increased production of IL-5 or G2Na non-neutralizing IgE antibodies, while IgG1- and IgG2-specific antibodies were boosted.

Harcourt et al. [94] reported on an increase in serum anti-RSV antibodies following a recent RSV infection of naïve children and adults vaccinated with live attenuated, cold-passage (cp), temperature-sensitive (ts) RSV vaccine. Such antibodies prevented the RSV challenge virus G glycoprotein from binding to the CX3CR1 receptors on leukocytes, preventing leukocyte chemotaxis by fractalkine. The authors wrote that ''one implication of these results is that humoral immunity to RSV infection or to vaccination may protect against immunomodulation effects associated with RSV G protein''.

Jin et al. [95] developed a cDNA clone of RSV A2 (15,222 nucleotides), but the production of infectious virus from the cDNA in transfected cells required co-transfection of three expression plasmids encoding RSV nucleoprotein (N), phosphoprotein (P) and the major polymerase protein (L). Inclusion of the M2-1 expression plasmid was not required. A recombinant RSV A2 strain that expressed an additional G glycoprotein from RSV subgroup B was also developed. Both A2 and B strains of G glycoproteins were expressed in cells infected with the chimera RSV, which is a live attenuated virus.

Whitehead et al. [96] reported on the development of ht rABcp248/404/1030 chimeric RSV and suggested that this chimeric virus is a promising vaccine candidate for RSV A and B subgroups, based on experiments in chimpanzees. The authors suggested that additional attenuating mutations derived from RSV strain A2 can be inserted into the A2 background of the recombinant chimeric RSV AB virus.

Jin et al. [97] removed NS1, NS2, SH and M2-2 genes individually and in different combinations from an infectious cDNA clone from human RSV A2 strain. The authors reported that (1) deletion of M2-2 with the NS1 gene was detrimental to virus replication; and (2) RSV recombinant A2 ΔM2-2 and NS2 was most attenuating, but the recombinant virus formed barely visible plaques in HEp-2 cells. All the deletion mutants were attenuated in the respiratory tract of cotton rats after intranasal inoculation. Similar results were obtained by Teng et al. [98] who tested the mutants in chimpanzees.

Spann et al. [99] co-infected HEp2 cells with two RSV recombinants lacking either the G gene (ΔG/HEK) or the NS1 and NS1/NS2 genes (ΔNS1/2). These viruses replicated inefficiently and produced pinpoint plaques in HEp-2 cells. The authors identified a larger plaque and found, by RT-PCR and sequencing of the viral genome, a polymerase jump from the ΔG/HEK genome to that of ΔNS1/1 genome and back to the vicinity of the SH-G-F genes by non-homologous and homologous recombination events, respectively, forming a short chimeric SH:G gene. The rec-RSV did not express the normal SH and G mRNA and proteins but expressed the aberrant SH:G mRNA.

The mechanism by which the FI-RSV vaccine immunization causes severe disease

Boelen et al. [100] aimed their study at characterizing the immunologic and inflammatory responses in BALB/c mice upon RSV infection with or without prior vaccination with aluminum-adjuvanted FI-RSV vaccine or uninfected FI-cell culture antigen (FI-Mock) as a control. It was reported that primary RSV infection in BALB/c mice resulted in a predominantly Th1-type cytokine response, which was associated with a slight bronchiolitis and alveolitis. In contrast, FI-RSV vaccination prior to RSV challenge prevented virus replication and was associated with aggravation of pulmonary histopathology and a shift toward Th2-type cytokine response. Nevertheless, vaccination with FI-Mock did not prevent the challenge RSV replication in the lungs and resulted in an even more pronounced Th2 cytokine response after RSV infection. The authors concluded that ''viral replication in the increased Th2 cytokine responding animal (induced by aluminum-adjuvanted mock vaccine) appears to boost the Th2 response upon RSV infection''.

Piedra et al. [101] studied the mechanism of cotton rat lung injury after vaccination with FI-RSV or FI-cell culture antigen (sham-immunized animals). RSV-negative cotton rats at 12 weeks of age were vaccinated with FI-RSV, live RSV and sham FI-cells and were challenged with 6×10^5 pfu/mouse of live RSV via the intranasal route. Animals were killed and evaluated for RSV antibodies, virus replication and pulmonary histopathology. It was reported that

(1) Of the animals that were immunized with FI-RSV, 88% developed anti-RSV neutralizing antibodies. RSV replication in the lungs was significantly reduced compared to FI, cell culture-immunized control rats. Both groups exhibited pulmonary histopathology, characterized by polymorphonuclear and mononuclear cell infiltrates.
(2) The virus-immunized animals manifested a more severe inflammatory reaction that reached a peak earlier than the virus-free, FI-cell culture-immunized control group.
(3) The sham-immunized animals that were infected with the live RSV developed little or no pulmonary histopathology.

The authors pointed to an inherent problem of the study, i.e. that live RSV and RSV vaccine preparations used in animal research are grown in *in vitro* cultured cell lines and not in human respiratory epithelial cells, the target cells of RSV in children and the elderly. The virus preparations were contaminated with cellular material and fetal calf serum that may contribute to pulmonary damage.

Johnson et al. [102] immunized BALB/c mice with RSV G glycoprotein or with FI-RSV and reported that after RSV challenge the mice exhibited severe disease, Th2 type cytokine production and pulmonary eosinophilia. RSV G-induced T cell responses were shown to be restricted to $CD4^+$ T cells expressing Vβ14 in T cell receptors (TCR). Depletion of this T cell subset resulted in a less severe disease. The authors examined the role of $V\beta14^+$ T cells in FI-RSV-induced disease. BALB/c mice were immunized with vaccinia virus expressing the secreted G glycoprotein (VVsG) or with FI-RSV vaccine. At the time of challenge with live RSV, mice were injected with antibodies to the Vβ14 component of TCR. It was reported that VVsG-immunized mice treated with anti-Vβ14 antibodies had reduced Th2 cytokine levels in the lung and reduced recruitment of eosinophils to the lungs. In contrast, depletion of $V\beta14^+$ T cells in FI-RSV-immunized mice had little impact on Th2 cytokine production and pulmonary eosinophilia. The authors concluded that ''although FI-RSV and VVsG induced similar immunopathology, the T cell response was different for each immunogen'' and that ''the immune responses elicited by RSV G are not the basis for FI-RSV vaccine-enhanced disease''.

Haynes et al. [103] reported that vaccination of BALB/c mice with RSV G glycoprotein lacking the CX3C FKN domain (GΔFKN) or RSV challenge of FI-RSV-vaccinated mice, or treatment with anti-substance P antibodies or antibodies to the CX3C domain, the antibodies reduced or eliminated the enhanced pulmonary disease, modified T cell receptor Vβ? usage, and altered CC and CXC chemokine expression. The authors indicated that ''the mechanisms by which CX3C–CX3CR1 interaction contributes to FI-RSV-enhanced disease are yet to be fully understood, but they may be linked to induction of pulmonary Substance P (SP) expression. Our data suggest that G glycoprotein induction of SP is linked to G glycoprotein CX3C–CX3CR1 interaction, and treatment with anti-SP antibody reduces G glycoprotein-associated enhanced pulmonary eosinophilia''.

Conclusion

Advances in research on the molecular biology of respiratory syncytial virus have led to an understanding of the mechanism by which the viral structural sG glycoproteins evade the host adaptive immune response and the viral NS1 and NS2 genes block the ability of infected respiratory epithelial cells to express the anti-viral interferon alpha and beta genes and thus produce interferons that will inhibit virus replication. In the absence of interferons, neighboring epithelial cells are not able to prevent the virus infection. In addition, it was reported that the sG glycoproteins contain two domains: the fractalkine (CX3C) motif and a superantigen domain. The function of the two sG motifs in the pathogenicity of RSV to infants, children and elderly people will be discussed and analyzed in the subsequent paper [104], in which the hypothesis that deletion of the fractalkine and superantigen domain from the G glycoprotein gene and NS1 gene may lead to an apathogenic RSV vaccine or RSV sGΔFKN, Sag glycoprotein vaccine is presented. Together with the adjuvant CpG ODN, immunization of humans may be possible.

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