THE ROLE OF MATERNAL ANTIBODIES IN RSV INFECTION

Emphasis on functionality and implications in disease

LIZ VAN ERP
The role of maternal antibodies in RSV infection

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PhD thesis, Radboud University, The Netherlands

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PART I

INTRODUCTION
RESPIRATORY SYNCTIAL VIRUS: DISCOVERY AND EMERGENCE

Respiratory syncytial virus (RSV) was identified for the first time in 1956 as the cause of mild respiratory illness in chimpanzees [1]. Not long afterwards, it was recognized to cause human airway infections with severe disease occurring mainly in infants [2]. The name of the virus was changed from chimpanzee coryza agent to respiratory syncytial virus, due to the characteristic of the virus to generate syncytial cells, which have been found in autopsy samples from fatal RSV infections and in tissue culture [2,3]. These syncytia are the result of fusion of infected cells with adjacent cells, leading to formation of multi-nucleated enlarged cells.

RSV is an enveloped virus with a single-stranded, negative-sense RNA genome, belonging to the Pneumoviridae family [4]. The RSV genome contains 10 genes encoding for 11 known proteins as is illustrated in Figure 1. Three of these viral proteins are transmembrane glycoproteins: the fusion protein (F), the attachment protein (G), and the small hydrophobic protein (SH) [5]. Both the F and G protein are often used as targets for monoclonal antibodies and vaccines. The RSV F protein has two conformational states: a pre- and post-fusion conformation. Most of the neutralizing activity in serum is thought to originate from pre-F specific antibodies [6].
**FIGURE 1** Respiratory syncytial virus (RSV) genome and virion.

A. The single-stranded, non-segmented, negative sense RSV genome, which contains 10 genes encoding 11 proteins. The M2 gene encodes for both M2-1 and M2-2 proteins.

B. The spherical morphology of RSV is shown here. RSV virions are pleomorphic and can also have a filamentous or asymmetric shape. The fusion (F), attachment (G), and small hydrophobic (SH) proteins are surface proteins, which are linked to the viral membrane. The nucleoprotein (N) protects the RNA from degradation and binds the RNA together with the large polymerase (L) protein and the phosphoprotein (P), which play a role in replication and transcription. The nucleoprotein is also present on the virion surface [7]. Transcription factors M2-1 and M2-2 regulate the switch between replication and transcription. In addition, M2-1 has a structural role and provides the link between the nucleoprotein and the matrix (M) protein, which lies underneath the viral membrane and provides the shape of the virion [8]. Not depicted in B are transcription factor M2-2 and the NS1 and NS2 proteins, two non-structural proteins that are thought to interfere with the host immune response.
Soon after its discovery, RSV was recognized as a leading global cause of respiratory disease in infants. RSV is a highly infectious virus, causing yearly epidemics during the winter months. In most individuals, RSV infection only leads to a common cold. However, in some cases, RSV can cause bronchiolitis and viral pneumonia, a result of excessive inflammation in the lower airways [9]. Hospitalization and mortality peak below six months of age, when most infants experience their primary RSV infection [10,11]. The estimated global incidence of RSV infections is 33.1 million cases per year, resulting in 2.3 million hospital admissions and as much as 118,200 deaths in children below 5 years of age [12]. In addition to infants, other vulnerable populations for severe RSV disease are the elderly, the immunocompromised, and individuals with underlying heart or lung conditions. Due to this substantial burden of disease, numerous vaccines and monoclonal antibodies are in (pre-) clinical development. Currently, the only market-approved drug to prevent RSV disease is Palivizumab, a prophylactic F protein-specific monoclonal antibody, which has been shown to reduce hospitalization in high-risk infants by 55% [13]. However, due to its high costs, the use of Palivizumab is restricted to children at high risk of RSV disease and its cost-effectiveness is under debate [14].

THE FORMALIN-INACTIVATED RSV VACCINE: IMPORTANT LESSONS FROM THE PAST

Development of new RSV vaccines has been hampered by the disastrous outcome of multiple trials with a formalin-inactivated (FI) vaccine in the 1960s [15-17]. Naïve vaccinees that received the FI-RSV vaccine were predisposed to enhanced disease upon a subsequent natural infection, which even resulted in the death of two children [15]. Since then, many research groups have tried to unravel the mechanisms underlying severe RSV disease. Although the precise cause of this vaccine–enhanced disease remains uncertain, it is thought that the induction of complement–binding, non-neutralizing antibodies in combination with a deleterious CD4+ T cell response are the main contributors [18–23]. The failure of these vaccine trials teaches us two important lessons: first, the immune response during an RSV infection is an important determinant of disease outcome; second, not just antibody levels, but also the functionality of the antibody response is crucial in preventing RSV disease.
THE ROLE OF MATERNAL ANTIBODIES IN RSV DISEASE

RSV hospitalization peaks below six months of age [10,11], when infants mainly rely on their innate immune system and maternal antibodies for protection against infectious diseases. Maternal antibodies predominantly consist of immunoglobulin G (IgG), as this is the only isotype that is transferred across the placenta in significant quantities [24]. Directly after birth, maternal antibody levels are high, which can provide partial protection against RSV infection in very young infants [25]. However, maternal RSV-specific antibodies quickly wane with a half-life that seems to vary by geographic location [26]. In western countries the maternal antibody half-life ranges from 21–26 days [27,28], in Bangladesh it was calculated to be 38 days [26], and a study in Kenya indicated a half-life of 79 days [29].

A NEW STRATEGY IN PROTECTION AGAINST RSV DISEASE: MATERNAL VACCINATION

Vaccine efficacy is low in infants below six months due to a poor T and B cell response resulting in an inadequate memory response [30]. An alternative strategy to protect infants at an early age is to vaccinate pregnant women. The rationale behind maternal vaccination is to boost RSV-specific antibody levels in the mother during pregnancy. These maternal antibodies will be transferred transplacentally to the unborn child, leading to higher RSV-specific antibody levels at birth. Maternal vaccines against pertussis, influenza, and tetanus are already approved and in use to protect both mother and child. No evidence of serious adverse events due to maternal vaccination have been reported so far [31,32]. However, before this vaccination strategy can be implemented to reduce the burden of RSV disease, we need more knowledge on RSV-specific maternal antibodies. In addition, the establishment of potential correlates of protection is essential for the evaluation of vaccine efficacy.

Many contradicting studies exist on the correlation between maternal antibody titers and protection against RSV infection or disease. Although high maternal antibody titers are associated with a reduced chance of acquiring (severe) RSV disease or infection in some studies [25,26,33,34], no clear correlate of protection is known. Multiple other studies could not detect a beneficial effect of high maternal antibody levels [35–38] or
even found an increased risk of recurrent wheezing [39]. However, the aforementioned studies only measured RSV-specific antibody levels or antibody neutralization capacity.

**AIM AND SCOPE OF THIS THESIS**

Considering that maternal vaccination is a potential strategy to prevent RSV disease in infants, it is of crucial importance to obtain a thorough understanding of the role of RSV-specific maternal antibodies in protection and disease. Previous studies have shown that the mere presence of a high virus-specific maternal antibody concentration does not ensure complete protection. In this thesis, we hypothesize that the functionality of RSV-specific antibodies plays an important role in RSV infection and disease. It is important to note that most conventional assays determine the antibody levels by *in vitro* binding or neutralization assays, while other antibody effector functions are not taken into account. In addition, maternal antibody levels wane quickly after birth. Therefore, the requirements to advance research into the role of RSV-specific maternal antibodies are two-fold: first, we need to develop assays to assess a broader variety of RSV-specific antibody features and effector functions; second, reliable models are required to investigate the effect of waning (local) antibody levels on RSV infection and disease severity.

In the first part of this thesis, we focus on Fc-mediated antibody effector functions, which are often overlooked in the current field of RSV research. An elaborate overview of the potential role of antibody functionality in RSV infection and disease is given in Chapter 2. We have developed multiple assays to study Fc-mediated antibody effector functions including antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent enhancement (ADE) of infection, and ADE of disease. In Chapter 3, we investigate both antigen-specific antibody levels and the capacity to induce NK cell activity of maternal antibodies from hospitalized RSV patients. Next, in Chapter 4, we use Fc gamma receptor-carrying cell lines to explore whether the capacity of antibodies to induce ADE of infection *in vitro* explains disease severity in hospitalized RSV patients and in a cotton rat FI-RSV model. In Chapter 5, we show that RSV can infect neonatal and adult natural killer (NK) cells, which is enhanced in the presence of sub-neutralizing antibody concentrations. Subsequently,
we investigate the impact of this infection on the effector functions of NK cells. Infection of NK cells is a common mechanism for viruses to evade the antiviral immune response. Chapter 6 describes all the viruses that are currently known to infect NK cells and the impact of infection on NK cell phenotype and effector function. In the last part of this thesis, we describe an in vivo mouse model to investigate the role of antibodies in RSV infection and disease. In Chapter 7, we compare different RSV inoculation methods, and use this knowledge to develop a mouse model for ADE of RSV infection and disease in vivo in Chapter 8. Finally, we discuss all our findings in Chapter 9 and elaborate on future perspectives.
REFERENCES

FC-MEDIATED ANTIBODY EFFECTOR FUNCTIONS DURING RESPIRATORY SYNCYTIAL VIRUS INFECTION AND DISEASE

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ABSTRACT

Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract infections and hospitalization in infants under 1 year of age and there is currently no market–approved vaccine available. For protection against infection, young children mainly depend on their innate immune system and maternal antibodies. Traditionally, antibody-mediated protection against viral infections is thought to be mediated by direct binding of antibodies to viral particles, resulting in virus neutralization. However, in the case of RSV, virus neutralization titers do not provide an adequate correlate of protection. The current lack of understanding of the mechanisms by which antibodies can protect against RSV infection and disease or, alternatively, contribute to disease severity, hampers the design of safe and effective vaccines against this virus.

Importantly, neutralization is only one of many mechanisms by which antibodies can interfere with viral infection. Antibodies consist of two structural regions: a variable fragment (Fab) that mediates antigen binding and a constant fragment (Fc) that mediates downstream effector functions via its interaction with Fc–receptors on (innate) immune cells or with C1q, the recognition molecule of the complement system. The interaction with Fc–receptors can lead to killing of virus–infected cells through a variety of immune effector mechanisms, including antibody–dependent cell–mediated cytotoxicity (ADCC) and antibody–dependent cellular phagocytosis (ADCP). Antibody–mediated complement activation may lead to complement–dependent cytotoxicity (CDC). In addition, both Fc–receptor interactions and complement activation can exert a broad range of immunomodulatory functions. Recent studies have emphasized the importance of Fc–mediated antibody effector functions in both protection and pathogenesis for various infectious agents.

In this review article, we aim to provide a comprehensive overview of the current knowledge on Fc–mediated antibody effector functions in the context of RSV infection, discuss their potential role in establishing the balance between protection and pathogenesis, and point out important gaps in our understanding of these processes. Furthermore, we elaborate on the regulation of these effector functions. Finally, we discuss the implications of Fc–mediated antibody effector functions for the rational design of safe and effective vaccines and monoclonal antibody therapies against RSV.
INTRODUCTION

Respiratory syncytial virus (RSV) infection is a major cause of severe respiratory illness requiring hospitalization in young infants [1]. Hospitalization for severe RSV-mediated disease peaks between six weeks and six months of life [2,3], when infants mainly depend on their innate immune system and maternal antibodies for protection against infectious diseases. However, the exact role of RSV-specific maternal antibodies is unclear. Some studies show that high titers of maternal antibodies are associated with protection against RSV infection [4–6]; whereas others indicate that high maternal antibody titers do not have a beneficial effect or even associate with an increased risk of recurrent wheezing [7–11]. It is important to note that the antibody titers in these studies are determined by in vitro binding or neutralization assays, while additional antibody effector functions are not taken into account.

For nearly all licensed vaccines, antibodies are the presumed correlate of protection, but the underlying mechanisms of protection often remain unknown [12]. Recent research suggests that, in addition to binding and neutralization, antibody effector functions are important contributors to protective immunity against several viruses, including influenza virus [13–15], HIV [16,17], and Ebola virus [18,19].

In contrast to their beneficial role in providing protection against infection and disease, antibodies have also been implicated in disease enhancement. For example, non-neutralizing dengue-specific antibodies have been shown to mediate antibody-dependent enhancement (ADE) of disease [20,21]. Interestingly, the 1960’s formalin-inactivated (FI) RSV vaccine induced poorly-neutralizing antibodies which have been suggested to be involved in vaccine-enhanced disease upon natural infection [22–24]. These examples illustrate the possibility that virus-specific antibodies contribute to pathogenesis when failing to protect.

Currently, the RSV field lacks a comprehensive overview of antibody effector functions in the context of RSV infection and disease. Here, we review what is known about various antibody effector functions during RSV infection, discuss their potential role in establishing the balance between protection and pathogenesis, and point out important gaps in our understanding of these processes. Moreover, we elaborate on the regulation of these effector functions on both the cellular and humoral side.
Finally, we discuss the implications of antibody-mediated effector functions for the rational design of safe and effective vaccines and monoclonal antibody therapies against RSV. A thorough understanding of the role of antibodies in protection or disease during RSV infection is crucial for the development of new and improved vaccination strategies and may provide much-needed new insights into the precise mechanisms of antibody-mediated protective immunity.

**FC-MEDIATED ANTIBODY EFFECTOR FUNCTIONS**

Antibody effector functions are an important part of the humoral immune response and form an essential link between innate and adaptive immunity. Most of these effector functions are induced via the constant (Fc) region of the antibody, which can interact with complement proteins and specialized Fc-receptors. The latter can induce activating or inhibitory pathways, depending on the type of receptor, and are found on B cells and most innate immune cells in various combinations. The most well-known Fc-mediated antibody effector functions are antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC). In addition, antibodies have been found to mediate inflammation and immunomodulation through the induction of cellular differentiation and activation. Each of these functions is described in detail below and a schematic overview is depicted in Figure 1.

**Antibody-dependent cell-mediated cytotoxicity (ADCC)**

ADCC is induced when Fc gamma receptors (FcγRs) on innate effector cells are engaged by the Fc domain of antibodies that are bound to viral proteins on the surface of virus-infected cells. This interaction induces the release of cytotoxic granules (containing perforins and granzymes) resulting in killing of infected cells [25]. Multiple innate effector cells, including natural killer (NK) cells, neutrophils, monocytes, and macrophages, are capable of ADCC in vitro. However, the most important contributors to ADCC in vivo are thought to be NK cells, which express only FcγRIIIA. Figure 2 shows a schematic representation of ADCC.

In the field of tumor immunology, ADCC has been recognized as an important mechanism of action for therapeutic monoclonal antibodies
(mAbs) that target tumor cells (as reviewed by [26]). For infectious diseases, ADCC only recently started to gain attention. ADCC has been shown to form a critical component of effective immunity against HIV and influenza virus. ADCC-inducing HIV-specific antibodies were identified as a key correlate of protection in the RV144 HIV vaccine trial [27–29]. Moreover, HIV-infected individuals who control the virus without antiretroviral therapy demonstrated a broader polyfunctional humoral immune response including ADCC activity compared to viremic individuals [30–33].

**FIGURE 1** Fc–mediated antibody effector functions

Antibodies elicit a wide range of effector functions during viral infections. These include but are not necessarily limited to the functions depicted in this figure. **Abbreviations:** DC, dendritic cell; FcyR, Fc gamma receptor; MAC, membrane attack complex; NK cell, natural killer cell.
There has been much debate about the role of ADCC during influenza-induced disease. Some studies point to the protective capacity of ADCC-inducing antibodies [34,35], whereas others do not show any role for NK cells in antibody-mediated protection [36], or even suggest involvement of ADCC in exaggeration of the immune response [37–39]. For multiple other clinically important viral infections, including dengue virus and Ebola virus, research into the effect of ADCC is ongoing [40–42]. Taken together, ADCC seems to be involved in the immune response against multiple viruses and is therefore potentially of interest in the context of RSV infection.

**ADCC in RSV infection**

NK cells are the most important contributors to ADCC in vivo and important effector cells during RSV infection. In mice, increased numbers of NK cells are present in the lungs early after RSV infection [43–45]. In RSV-infected infants, the proportion of NK cells has been reported both to be decreased [46–49] or increased [50,51] in comparison with healthy controls or infants.
with mild symptoms. Since maternally-derived antibodies are virtually always present during primary RSV infection and antibody-coated virus-infected cells are a trigger for ADCC, it can be assumed that ADCC occurs during primary RSV infection.

Although NK cells are thought to be the most important mediators of ADCC against virus-infected cells, this has never been shown for RSV. All studies mentioned below are performed with peripheral blood mononuclear cells (PBMCs), without distinction between different cell types. RSV-specific immunoglobulin G (IgG) has been shown to induce ADCC towards RSV-infected epithelial cells in vitro [52,53]. The major surface antigens of RSV are the fusion (F) and the attachment (G) protein which are both required for infectivity in vivo. The RSV F protein has two conformational states: post-fusion (post-F) and pre-fusion (pre-F), of which the latter is a potent target for neutralization [54]. Multiple studies show that anti-RSV G antibodies are efficient inducers of ADCC in vitro [55,56], and the involvement of this process in virus clearance in vivo has been proposed [57,58]. In contrast, anti-RSV F antibodies do not efficiently induce ADCC in vitro [55], although it must be noted that no distinction between pre- and post-F antibodies was made and the ADCC potential could differ between the two functional states of the F protein.

Antibodies from breast milk, cord blood, and nasopharyngeal secretions and serum from RSV-infected infants show ADCC activity in vitro [52,53,59]. This shows that the antibodies that are present in vivo are capable of eliciting ADCC activity in vitro. Two studies showed that the level of ADCC activity measured in vitro was independent of clinical symptoms and age, suggesting that ADCC is not a determining factor in the varying clinical manifestations of primary RSV infection [53,59]. Interestingly, the ADCC capacity of serum antibodies from RSV-infected infants rapidly declines over time, whereas the neutralization capacity remains more stable. If ADCC is important in protection against infection, this decline could partly explain the susceptibility to repeated infections throughout life.

Limited evidence is present on the occurrence of ADCC during RSV infection in vivo. The most convincing data is provided by mouse studies performed with anti-RSV G protein-specific Fab- or F(ab’)2 fragments lacking the complete Fc domain, or aglycosylated antibodies lacking the glycosylation site that is required for efficient FcyR and complement interactions [58,60,61]. It was shown that Fab fragments of the 1812A2B
anti–RSV G antibody and F(\(\text{ab}'\))\(_2\) fragments of the 131–2G anti–RSV G antibody do not reduce viral load, whereas the corresponding intact antibodies do confer protection [58,60]. The authors propose that virus clearance by the 131–2G antibody is mediated through ADCC, however, the involvement of other Fc–mediated effector functions in this study cannot be ruled out. In an attempt to ascertain the role of ADCC by NK cells in the protective mechanisms of the anti–RSV G antibody 18A2B2, SCID beige mice (which are deficient in NK cell activity) were passively immunized with the full antibody [60]. In this study, the absence of NK cells had no effect on the protective capacity of 18A2B2, pointing to the involvement of other Fc effector functions. Further research is needed to study the exact role of ADCC for other mAbs and RSV–immune serum. Passive immunization with aglycosylated 1C2 anti–RSV G antibodies reduced virus titers significantly but were not as effective as wildtype antibodies, indicating that protection by the 1C2 antibody is mediated by both Fc–dependent and Fc–independent mechanisms [61]. Although these studies highlight the importance of Fc–mediated antibody effector functions in protection against RSV infection in the case of these specific anti–RSV G mAbs, the role of ADCC in protection or pathogenesis during natural RSV infection remains to be determined.

Antibody–dependent cellular phagocytosis (ADCP)

ADCP or opsonophagocytosis is the uptake of virus–antibody complexes or antibody–coated virus–infected cells by phagocytic cells (for a schematic representation of this process see Figure 3). Phagocytic cells, including monocytes, macrophages, neutrophils, eosinophils and dendritic cells (DCs), express FcγRI, FcγRII, and FcαRI, which can all mediate immune complex uptake. The exact phagocytic capacity of effector leukocytes is dependent upon the cell type, differentiation stage, and level of FcγR expression. ADCP results in the clearance of immune complexes from the infected host, by trafficking of the complexes to lysosomes for degradation and antigen processing for presentation on Major Histocompatibility Complex (MHC)–molecules on the cell surface. Interestingly, some viruses have exploited this mechanism to infect phagocytes by escaping from lysosomal degradation (described below in “Antibody–dependent enhancement of infection”).

ADCP has been extensively described for its role in protection against
bacteria, but its importance during viral infections is unclear. Some studies have been performed for influenza virus, showing that phagocytosis by (alveolar) macrophages may contribute to protection from infection in mice [36,62] and potentially plays a role in the recovery from severe infections in humans [15,63]. Also for cytomegalovirus (CMV), it was shown that vaccine-induced antibodies play an important role in vaccine efficacy, independent of neutralization or ADCC capacity [64]. In accordance with these results, a study by Nelson et al. showed no role for neutralization or ADCC, while robust ADCP induction was observed [65]. Antibody-mediated clearance by phagocytes in vivo has also been suggested for HIV [66,67], adenovirus [68], West Nile Virus (WNV) [69], and foot-and-mouth disease virus [70,71].

**ADCP in RSV infection**

Phagocytosis of RSV-antibody complexes or RSV-infected cells has to our knowledge never been directly explored as a protective immune mechanism for RSV. *In vitro* studies show phagocytosis of RSV immune complexes by neutrophils [56,72,73] and eosinophils [74]. Varying levels

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**FIGURE 3** Antibody-dependent cellular phagocytosis (ADCP).

Phagocytes can clear virus-infected cells and immune complexes that are engaged by Fc gamma receptors through phagocytosis. Uptake of viral particles or proteins leads to antigen presentation, which induces the adaptive immune system. **Abbreviations:** FcyR, Fc gamma receptor; MHC, major histocompatibility complex; RSV, respiratory syncytial virus.
of phagocytic activity have been observed for different RSV-specific monoclonal antibodies, suggesting that ADCP activity depends on epitope and/or affinity [56,73]. An in vivo mouse study has shown that macrophages are essential in conferring antibody-mediated restriction of RSV replication, whereas neutrophil depletion did not significantly affect pulmonary viral replication [75]. This suggests that Fc-mediated effector functions executed by macrophages rather than neutrophils are important in protection against RSV infection in a mouse model.

Besides the uptake of viral particles, phagocytosis initiates the activation of cells. This can result in the release of a broad range of effector molecules [72-74], which will be described in detail in “Antibody-dependent immunomodulation during RSV infection”. Although there is limited evidence on the role of ADCP during RSV infection, the importance of macrophages in antibody-mediated protection in mice provides a basis for further investigation.

**Antibody-mediated complement activation**

Besides ADCC and ADCP, antibodies can also induce complement activation. The complement cascade contributes to pathogen elimination either directly, by means of complement-dependent cytotoxicity (CDC), or indirectly, through phagocytic clearance of complement-coated targets and the induction of an inflammatory response. Activation of the classical complement pathway results from binding of the recognition molecule C1q to the Fc domain of antibodies bound to virus-infected cells [76,77], as depicted in Figure 4. Upon binding of C1q, the proteases of the classical pathway are activated, leading to cleavage of C2 and C4. Together, the resulting cleavage products form the C3 convertase (C4bC2a) that cleaves C3 into C3a and C3b. One of the mechanisms by which the complement cascade is regulated, is cleavage of active C4b, which serves as a marker for complement activation. The release of anaphylatoxins C3a and C5a stimulates a pro-inflammatory environment by inducing the recruitment of immune effector cells and the activation of leukocytes, endothelial cells, epithelial cells, and platelets [78,79]. The highly reactive C3b binds to pathogens and infected cells, leading to immune complex clearance and phagocytosis through complement receptors found on immune cells. The terminal complement components will assemble into the membrane attack complex (MAC), resulting in lysis of the infected cell. Besides direct
antiviral activity, the complement system can also regulate B cell responses. The binding of complement-coated immune complexes to complement receptor 2 on B cells is reported to lower the B cell activation threshold, thereby promoting long-lived adaptive immunity and higher antibody levels [80,81]. Complement can have both a protective and pathogenic role during viral infections. The protective capacity of poorly neutralizing antibodies during

**FIGURE 4** Antibody-mediated complement activation

Binding of C1q to antibody-bound virus-infected cells leads to activation of the classical complement pathway. C3 convertase (C4bC2a) is formed and cleaves C3 into C3a and C3b. Active C4b can be cleaved into the enzymatically inactive form C4d, which serves as a marker for complement activation. Further downstream in the classical complement pathway, C5 is cleaved into C5a and C5b. C3a and C5a are anaphylatoxins that stimulate a pro-inflammatory environment, although they act in different ways: C3a induces C3aR signaling, whereas C5a inhibits C3aR expression. C3b binds to pathogens and infected cells, leading to phagocytosis through complement receptors found on immune cells. The terminal complement components will assemble into the membrane attack complex (MAC), resulting in direct lysis of the infected cell. **Abbreviations:** C3aR, C3a Receptor; MAC, membrane attack complex; RSV, respiratory syncytial virus.
WNV infection is mediated by the complement system, as was shown using knockout mice [69]. The presence of complement even enhances the neutralization capacity of WNV-specific antibodies [82]. In addition, an important role for complement has been shown in the protective capacity of (monoclonal) antibodies against influenza virus [39,83], vaccinia virus [84], CMV [85], and HIV [66,67]. In contrast, complement activation has also been suggested to contribute to disease severity in dengue virus [86,87] and HIV infection [88,89].

Antibody-mediated complement activation in RSV infection
The complement system consists of multiple components and elicits its effector functions through different pathways. Early studies have shown antibody and complement deposition on nasopharyngeal cells of RSV-infected infants [90]. Whether this contributed to viral clearance or disease was not determined. Studies in complement-deficient mice have shown that complement is important in antibody-mediated protection against RSV infection [60,75]. A number of different mechanisms have been suggested for this complement-enhanced protection. Firstly, direct enhancement of the neutralization capacity of antibodies by fixation of complement components to virus-antibody complexes may increase the steric hindrance of bound antibodies [91]. Another mechanism that could be at play is complement-dependent opsonization of virus-infected cells, which leads to subsequent uptake by phagocytes. Finally, complement has also been shown to increase the CD4(+) T cell response in the presence of RSV immune serum in an in vivo mouse model [92].

Besides its potential role in the clearance and/or pathogenesis of natural RSV infection, complement activation has been suggested to contribute to disease enhancement induced by natural infection following FI–RSV vaccination. C3a receptor (C3aR)-deficient mice had decreased airway hyperresponsiveness (AHR) and less mucus production in an FI–RSV vaccination–challenge model [93]. In this study, C3aR expression was enhanced in C5–knockout mice, showing that the balance in activation of different complement factors (C3a versus C5a) is important in determining disease outcome. Moreover, Polack et al. demonstrated the co-localization of IgG and C3 in the lungs of mice with enhanced RSV disease, but not in control mice [22]. In addition, both C3– and B cell–knockout mice showed a decrease in bronchoconstriction compared to WT mice vaccinated
with FI-RSV. Therefore, in a mouse model of vaccine-enhanced disease, the presence of C5 seems protective, whereas C3a promotes enhanced disease. This is also supported by the limited data available on complement activation during vaccine-enhanced disease in infants. Lung sections of the two children who died of vaccine-enhanced disease had extensive deposition of complement cleavage product C4d, which serves as a stable marker for complement activation [22]. The presence of C4d provides evidence for complement activation during vaccine-enhanced disease in infants, but it remains to be determined whether there is a causal relation between complement activation and vaccine-enhanced disease. Finally, mouse studies point to the involvement of complement components in the development of AHR and asthma upon RSV infection [94,95]. Taken together, the complement system seems to be important in antibody-dependent protection in vivo, but it also potentially contributes to (vaccine-enhanced) disease and asthma, suggesting a dual role in RSV infection that requires further investigation.

**FIGURE 5** Antibody-mediated immunomodulation

Immune complexes can skew immune cell maturation and activation of granulocytes, dendritic cells, T cells, B cells, and phagocytes. This immunomodulation can lead to

- A. degranulation
- B. skewing of T cell responses
- C. regulation of B cell antibody responses
- D. phagocytosis-induced secretion of immunomodulatory mediators

Abbreviations: ROS, reactive oxygen species.
Antibody-mediated immunomodulation

Besides the well-defined classical Fc-mediated effector functions (ADCC, ADCP, CDC), immune complexes can also promote immune cell maturation and activation, leading to a wide range of effector activities and production of pro-inflammatory and immunomodulatory mediators (a limited overview is depicted in Figure 5). Some of these pro-inflammatory cytokine responses correlate with protection as has been shown for influenza [62] and HIV [96]. The importance of FcγRs in this process has been shown by the use of FcγR-deficient mice (as extensively reviewed in [97]). In contrast to the pro-inflammatory responses caused by immune complexes, injection with intravenous immunoglobulin (IVIg) can induce an anti-inflammatory state. It is proposed that this anti-inflammatory effect is partly due to the presence of sialylated antibodies in IVIg, which induce expression of FcγRIIB (the only inhibitory FcγR) and thereby dampen the inflammatory response [98].

Immune complexes can also regulate cellular maturation and activation. The balance between inhibitory and activating FcγR interactions is crucial in regulating B cell IgG responses [99-101], and skewing APC maturation and antigen presentation [102-105], which can modulate T cell activation. Immune complexes have also been shown to bias the macrophage immune response towards a Th2-like phenotype [106].

Antibody-mediated immunomodulation in RSV infection

RSV–antibody complexes can lead to activation of phagocytes either directly or after phagocytosis, resulting in the production of reactive oxygen species (ROS), thromboxane, (pro-inflammatory) cytokines, and chemokines [72,73,107], which may contribute to viral clearance. However, these mediators can also have immunopathological effects, including tissue damage, platelet aggregation, and bronchoconstriction. Given that neutrophils are the predominant airway leukocytes present in RSV-infected infants, their activation is suggested to be involved in the induction of severe RSV disease [108]. Interestingly, in contrast to RSV immune complexes, it has been reported that RSV alone does not lead to ROS production by granulocytes [107] and can even inhibit this process [73,109]. It has been suggested that anti-RSV G mAbs are less potent inducers of ROS and cytokine production than anti-RSV F mAbs [73], but this was based on experiments with only two RSV–specific antibodies. Notably, differences
in the capacity to induce a response may not be due to antigen-specificity per se but rather due to epitope localization, as described in the paragraph “Important epitopes during RSV infection”.

Excessive eosinophilic activation has been suggested to play a role in the immunopathology of FI-RSV-induced disease in mice [22]. Whether the non-neutralizing antibodies induced by the FI-RSV vaccine play a role in this activation remains unknown. In vitro studies have shown that eosinophils can phagocytose RSV-antibody complexes, leading to degranulation [74]. The use of heat-inactivated serum abolished this effect, indicating complement involvement.

Besides an immunomodulatory effect on granulocytes, RSV-antibody complexes can also affect T cell responses. Kruijsen et al. show in an in vivo mouse model that IFN-γ secretion by CD4(+) T cells is increased in the presence of RSV immune serum [92]. This increase is dependent on both FcyRs and the complement system. Additional in vitro experiments indicate that both anti-RSV G, as well as anti-RSV F antibodies can induce this enhanced CD4(+) T cell response, whereas CD8(+) T cells are only activated by the presence of anti-RSV G antibodies. Another in vitro study found that DCs primed with complexes composed of RSV and F-specific antibodies displayed an impaired capacity to activate CD8(+) and CD4(+) T cells [110].

RSV-antibody complexes also contribute to antibody-mediated immunomodulation through the induction or inhibition of cytokine and chemokine production in PBMCs. In an in vitro study, RSV-antibody complexes inhibited IFN-α production in PBMCs, whereas these complexes enhanced IFN-α production of PBMCs in the absence of CD14(+) cells [111]. Another in vitro study showed that, compared to RSV alone, RSV immune complexes induce increased IFN-α, IFN-γ, CXCL10, and CXCL11 production in monocytes [112]. In infant PBMCs, only CXCL10 production was significantly enhanced. CXCL10 can mediate a neutrophil-dependent excessive pulmonary inflammation [113], which could contribute to RSV pathogenesis. This indicates that immune complexes can potentially also activate neutrophils indirectly, through the induction of chemokines and cytokines in PBMCs. Altogether, these studies show that immune complexes are able to skew the RSV-specific immune response in multiple ways, but more research is needed to clarify the exact contribution of antibody-mediated immunomodulation to protection and disease during RSV infection.
Antibody–dependent enhancement (ADE) of infection

ADE refers to a phenomenon in which virus–specific antibodies promote, rather than inhibit, infection and/or disease. In ADE of infection, also known as extrinsic ADE [114], the number of virus–infected cells is increased in the presence of (natural or monoclonal) antibodies that are non–neutralizing or present in sub–neutralizing concentrations. ADE of infection requires the presence of FcγRs on target cells and is an efficient in vitro tool to assess Fc–FcγR interactions. However, while ADE of infection has been observed for many viruses in vitro (as extensively reviewed in [115]), its significance in vivo remains uncertain. A schematic representation of ADE of infection is depicted in Figure 6.

ADE of RSV infection

ADE of RSV infection has been demonstrated in vitro for both mAbs and RSV–immune serum in monocytic cell lines, PBMCs, neonatal and adult NK cells, and primary mouse and cotton rat immune cells [110, 116–120].

**FIGURE 6** Antibody–dependent enhancement (ADE) of infection

ADE of infection has been shown in vitro for multiple viruses, including RSV. High antibody titers neutralize the virus completely. Sub–neutralizing antibody titers form immune complexes that can interact with both the virus receptor and Fc gamma receptors, leading to enhanced infection levels compared to infection in the absence of antibodies.
However, whether the ADE of infection observed *in vitro* is related to *in vivo* disease outcome is doubtful. No correlation has been found between disease severity in infants and the capacity of serum antibodies to induce ADE of RSV infection *in vitro* [119]. Furthermore, ADE of infection has never been demonstrated *in vivo*. However, it must be noted that this has never been assessed during FI–RSV vaccine–enhanced disease.

**Antibody-dependent enhancement (ADE) of disease**

ADE of disease, or intrinsic ADE [114], refers to a process in which the presence of pathogen–specific antibodies contributes to disease severity. For example, immune complexes might bind to FcγR-expressing immune cells, modulating the immune response and subsequently leading to enhanced inflammation. ADE of disease has been a presumed cause of severe disease following various viral infections and vaccinations [37, 114, 121–123]. However, the underlying mechanisms are largely unknown and *in vivo* data supporting these claims are often lacking. However, for dengue virus infection some first clues to unravel the mechanism underlying ADE of disease have recently been published. Wang *et al.* have been able to show a correlation between FcγRIIIA binding capacity of dengue virus antibodies and disease severity *in vivo* [20]. The dengue-specific antibodies are thought to cross-react with platelet antigens and induce thrombocytopenia. Suggested underlying mechanisms are FcγR-mediated platelet activation, phagocytosis or ADCC, but further investigation is needed to confirm these hypotheses. In addition, Katzelnick *et al.* have shown that high dengue-specific antibody titers correlate with protection, whereas intermediate antibody titers correlate with severe dengue disease [124]. Although low or no antibody titers are not protective, they do not enhance disease. It is possible that RSV-specific antibodies show a similar pattern, as illustrated schematically in Figure 7.

ADE of RSV disease

Although *in vitro* ADE of infection does not seem to be a determinant for severe RSV disease [119], other antibody–mediated mechanisms could be involved, as has recently been shown for dengue virus infection [20]. Many animal studies on RSV infection highlight the role of an excessive immune response in FI–RSV vaccine–enhanced disease. It is likely that poorly-neutralizing vaccine–induced antibodies play a role in the development of
FI–RSV vaccine–enhanced disease [22–24], although it remains uncertain which Fc-mediated effector functions are involved. Little is known on the involvement of (maternal) antibodies in the development of severe disease after natural RSV infection. Severe RSV infections are most frequently seen in the first 6 months of life when infants have circulating maternal RSV–specific antibodies [2]. This suggests that RSV–specific antibodies may contribute to the induction of severe RSV disease. Results from animal studies with Fab fragments and FcγR–knockout mice indeed show the involvement of antibody–mediated effector functions both in protection against viral replication [58,60,61] and in promoting inflammation [92]. Some studies have reported enhanced RSV disease to occur in the
presence of waning immunity. Murphy and colleagues reported enhanced pulmonary pathology 3 months after immunization with an RSV F glycoprotein vaccine [125], which was not seen 1 week after immunization [126]. In a follow-up study, enhanced lung pathology was observed upon immunization with low doses of recombinant F protein, mimicking waning immunity [127]. Interestingly, the enhanced disease was independent of the presence of a Th1- or Th2-biasing adjuvant.

Taken together, there are clear indications suggesting that Fc-mediated antibody effector functions may contribute to severe RSV disease. Complement activation has been linked to vaccine-enhanced disease and asthma, and may therefore also be involved in severe RSV disease upon natural infection. In addition, the immunomodulatory effects of immune complexes can lead to a pro-inflammatory environment, which is thought to be the underlying cause of RSV-mediated pathology. However, more research on the involvement of individual Fc-mediated effector functions in disease outcome following RSV infection is needed.

**REGULATION OF Fc-MEDIATED EFFECTOR FUNCTIONS**

Fc-mediated antibody effector functions play an important role in shaping the immune response and their active regulation is crucial to prevent excessive immune activation. A number of determinants have been found to influence Fc-mediated effector functions on both the cellular and antibody side of the Fc–Fc receptor (FcR) interaction. Important antibody characteristics are the isotype, subclass, glycosylation pattern, and antigen specificity, while important cellular determinants are the epitope position relative to the target cell membrane and FcR expression and polymorphisms on the effector cell, which together determine the capacity of the antibody to interact with specific FcRs. Most antibodies are not specifically eliciting a single effector function, and therefore the combination of all these characteristics determines the outcome of the various Fc–FcR interactions and the interaction with the complement system.

**Antibody isotype and subclass**

Antibodies consist of two functional domains: the variable antigen-binding fragment (Fab) and the constant fragment (Fc) that interacts with FcRs and C1q. The isotype of the Fc domain (IgA, IgD, IgE, IgG, and IgM) represents
the major determinant of Fc-mediated effector functions. Of these isotypes, IgG is the most important when it comes to Fc-mediated effector functions, as this is the only isotype known to interact with the widely expressed FcγRs. Whereas the majority of antibodies in serum are of the IgG subtype, IgA is the major isotype present in mucosal secretions. This isotype interacts with its specific receptor FcαRI, which is present on neutrophils, eosinophils, monocytes, and macrophages (extensively reviewed in [128]). Activation of FcαRI by IgA-opsonized pathogens can induce ADCC, phagocytosis, degranulation, and cytokine release. Other important isotypes to briefly mention are IgM, which is a potent complement activator [76], and IgE, which has been linked to various allergic diseases.

In humans, four different IgG subclasses (IgG1–IgG4) are known. These subclasses differ in amino acid sequence, which influences their capacity to interact with certain classes of FcγRs and complement components as depicted in Table 1. Production of different isotypes and subclasses is tightly regulated and dependent on differentiation of the B cell, which can be influenced by cytokines and interactions with pattern-recognition receptors. The response to protein antigens usually involves T cell help and induces class switching to IgG1 or IgG3, whereas polysaccharide antigens induce class switching to IgG2 in the absence of T cell help [129]. Viral

### TABLE 1  Functionality of IgG subclasses

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Serum abundance</th>
<th>FcγRI</th>
<th>FcγRIIa</th>
<th>FcγRIIb</th>
<th>FcγRIIIa</th>
<th>FcγRIIIb</th>
<th>C1q</th>
<th>Effector functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>60 %</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>ADCC, ADCP, CDC</td>
</tr>
<tr>
<td>IgG2</td>
<td>32 %</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IgG3</td>
<td>4 %</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>ADCC, ADCP, CDC</td>
</tr>
<tr>
<td>IgG4</td>
<td>4 %</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; FcγR, Fc gamma receptor; IgG, immunoglobulin G [133-135].
infections, including RSV infections, mostly induce IgG1 and IgG3 antibody responses [130–132].

IgG1 and IgG3 have the highest affinity for FcγRs and are potent activators of complement, ADCC and phagocytosis [133,136,137]. IgG3 is the subclass with the highest potential to activate both FcγRs and complement, but due to its short half-life the preferred subclass for therapeutic cytotoxic activity is IgG1 [138]. In contrast, receptor-blocking antibodies are often of the IgG2 or IgG4 subclass to avoid Fc-mediated cytotoxic side effects [139]. Induction of specific subclasses can have major effects on the outcome of vaccine trials as has been shown for the HIV RV144 and VAX003 vaccines. RV144 recipients produced highly functional IgG3 antibodies that provided 31.2% efficacy, whereas VAX003 recipients elicited a monofunctional IgG4 antibody response that was not protective at all [140].

*Antibody isotype and subclass in RSV infection*

Severe RSV-mediated disease is most prevalent in infants below 6 months of age. These children mainly rely on maternally-derived IgG for protection against infectious diseases, but the correlation between serum IgG levels and protection against RSV disease is poor [8–11]. A recent study by Habibi et al. found that mucosal IgA titers are a better predictor of susceptibility to RSV infection than serum IgG levels in an adult challenge model [141]. In addition, they showed a hampered IgA memory B cell response to RSV, which may explain the life-long susceptibility to repeated RSV infections. In accordance with these results, lower levels of nasal IgA were found in naturally RSV-infected adults compared to healthy controls [142]. These findings highlight the importance of mucosal IgA in protection against RSV infection. However, it is questionable whether IgA plays a role in protection or disease during primary infection. IgA antibodies to RSV are only found in secretions after 4 months of age, confirming they are synthesized as a result of (primary) infection [143]. RSV-specific IgA has been shown to induce antibody-mediated effector functions. Although palivizumab–IgA demonstrated slightly higher lysis of RSV-infected HEp2 cells by neutrophils (but not monocytes) in vitro, there was a somewhat decreased efficacy in vivo compared to palivizumab–IgG [144]. Additional experiments with FcαRI transgenic mice suggest that IgA-mediated protection is Fc receptor-independent. No further research with RSV–IgA immune complexes has been published to date and therefore their role in
Another interesting isotype is IgE, as the results from multiple studies suggest the involvement of this isotype in the development of RSV-mediated bronchiolitis and wheezing [145-148]. In a mouse model, RSV–specific IgE has been shown to enhance airway hyperresponsiveness [149]. Since all infants produce IgE in response to RSV infection [150], it is thought that the height and duration of the IgE response are important for the induction of subsequent immunopathology [148, 151, 152]. Mast cells abundantly express the IgE-specific Fc receptor (FcεRI) and were shown to play an important role in IgE-induced airway hyperresponsiveness in an RSV reinfection mouse model [149].

In addition to studies on isotypes, extensive studies have been performed on the presence of IgG subclasses during RSV infection. Wagner et al. have performed some early studies into the antibody subclass response to the RSV F and G glycoproteins in both infants and adults [131, 153, 154]. Primary RSV infections predominantly gave rise to IgG1 and IgG3 antibodies, whereas subsequent infection only led to an increase in IgG1 and IgG2 titers [131]. RSV infection led to a poor IgG4 antibody response in all subjects. RSV F protein was the most immunogenic, leading to higher antibody titers compared to the RSV G protein [153]. The IgG1/IgG2 ratio of antibody titers to the RSV F protein was fourfold higher than to the RSV G protein after the first three RSV infections in infants. This difference was thought to be due to the extensive glycosylation of the G protein, resulting in IgG2 antibodies. IgG1 and IgG3 are the most potent FcγR-binding subclasses. This suggests that the majority of anti-RSV F antibodies are effective inducers of Fc–mediated effector functions, in contrast to the IgG2 subset of anti-RSV G antibodies. Experimental RSV infection in adults showed similar subclass responses to RSV F and G protein [154]. A recent study confirms the findings of Wagner et al., showing a strong IgG3 response in infants younger than 4 months, despite the presence of high levels of maternal antibodies [155]. A rise in RSV–specific IgG1 and IgG2 was only observed in infants older than 7 months.

Besides human studies, several mouse studies have been performed to investigate the subclass antibody response. Although some homology between mouse and human IgG subclasses has been found, it is unclear whether they induce the same downstream immune responses. In mice, neonatal IgG responses to RSV infection are significantly skewed towards
mlgG1 (homologous to human IgG4), indicating a Th2 bias [156], whereas primary infection in adult mice leads to a balanced mlgG2a/mlgG1 response (homologous to human IgG1/IgG4) [157]. Compared to wild-type RSV infection, immunization with inactivated or nonreplicative RSV led to a low mlgG2a/mlgG1 ratio [24, 158]. The largest proportion of antibodies directed at the RSV–F protein was mlgG2 (homologous to human IgG1), whereas the G protein response had a significantly lower proportion of mlgG2 [158]. These results indicate that both the age of the host and the antigens determine the subclass response. However, it is remarkable that RSV infection leads to a poor IgG4 antibody response in humans, but to a high mlgG1 (homologue of human IgG4) response in (neonatal) mice. Thus, caution is warranted in the translation between human and mouse antibody studies.

Although extensive studies have been performed on the presence of specific subclasses, evidence on the role of these different subclasses during RSV infection is limited. One study describes a direct comparison between the functionality of palivizumab–IgG1 and –IgG2 [159]. The neutralizing potential of both subclasses was comparable. However, the IgG2 antibody showed negligible binding to murine FcγRs and human C1q, resulting in less efficacy in vivo as measured by increased viral lung titers in challenged cotton rats [159]. This finding underscores the protective potential of IgG1–mediated effector functions during RSV infection.

**Antibody glycosylation**

Glycosylation of the antibody Fc domain is another important regulator of Fc–mediated effector functions. Each IgG molecule contains a highly conserved asparagine at position 297 (N297) that functions as a glycosylation site that can harbor a variety of glycans, consisting of varying combinations of mannose, (bisecting) N-acetylglycosamine (GlcNAc), fucose, galactose and sialic acid (Figure 8). The complete absence of this glycan leads to a conformational state that is non-permissive for FcγR or complement binding, thereby impairing Fc–mediated antibody effector functions.

Afuicosylation has the most straightforward influence on antibody effector functions. The absence of the core fucose on the Fc–glycan directly boosts ADCC activity by enhancing the interaction with FcγRIIIA (Figure 2) [160–162]. Interestingly, afucosylated mAbs have shown to be more protective against various infectious agents [163,164] and more efficacious in cancer.
therapy [165,166]. However, increased levels of afucosylation are also associated with severe disease during secondary dengue infection [20].

Another biologically important modification to the Fc glycan is sialylation. The presence of sialic acid inhibits FcγR binding and is reported to be partly responsible for the anti-inflammatory activity of IVIg [98,167]. Besides having anti-inflammatory properties, sialylated Fc glycans have also been shown to induce the production of high-affinity broadly neutralizing antibodies against influenza virus [101].

Besides its effect on Fc receptor interactions, Fc glycosylation also affects complement C1q binding to immune complexes. A recent study shows that elevated galactosylation and sialylation increase C1q-binding, downstream complement deposition, and complement dependent cytotoxicity [168]. In contrast, agalactosylated IgG has also been suggested to elicit enhanced complement activation, considering its role in several autoimmune diseases [169]. These findings suggest that activation of complement potentially contributes to pathogen clearance, but can also contribute to inflammation in autoimmune disease, highlighting the dual role of complement.

Fc glycosylation is subject to active regulatory mechanisms that control the composition of the glycan structure. Major changes in glycosylation

**FIGURE 8** Antibody glycosylation

Each IgG molecule contains a glycosylation site that can harbor a variety of glycans, consisting of varying combinations of mannose, (bisecting) N-acetylglycosamine, fucose, galactose and sialic acid. Antibody effector activity is substantially impaired in absence of this glycan. **Abbreviations:** Fab, antigen-binding fragment; Fc, crystallizable fragment; GlcNAc, N-acetylglycosamine.
occur during pregnancy [170,171], upon vaccination [101,172], and during certain viral infections [101]. Therefore, insight in the glycosylation pattern during RSV infection and disease may provide valuable clues on the cause of severe RSV disease.

Antibody glycosylation in RSV infection
To our knowledge, only one group has investigated the effect of glycosylation in the response towards RSV infection. Hiatt et al. compared the original Palivizumab mAb with an afucosylated and agalactosylated plant–produced glycovariant (G0) in different in vitro and in vivo assays [159]. The G0 glycovariant showed enhanced binding to murine FcγRs but less binding to human C1q compared to the parental Palivizumab, whereas neutralization capacity was comparable. The in vivo protective capacity of the G0 glycovariant was improved compared to the original, as evidenced by decreased pulmonary viral titers. In conclusion, this study suggests that the influence of Fc-glycosylation may be important in the protective capacity of RSV-specific antibodies but this needs to be studied in more detail for other mAbs and virus– and vaccine–induced antibodies.

Epitope position
Next to antibody structure and glycosylation, the location of the antibody–bound epitope with respect to the membrane of the infected cell has been shown to be pivotal in determining Fc–mediated effector functions. Since the use of mAbs, it has been noticed that different mAbs binding the same target protein can elicit different effector mechanisms [173]. Antibodies binding to epitopes closer to the membrane (membrane proximal epitopes) mediate ADCC and CDC activity more efficiently, whereas antibodies that target membrane distal epitopes are often highly neutralizing and efficient ADCP–inducers [13,174,175]. More specifically, recent research suggests that ADCP is most efficiently triggered when antibodies bind within 10 nm from the cell surface [176], indicating that the optimal ADCP–inducing epitope is located neither too close, nor too far away from the cell membrane. These studies suggest that besides the common need for particular Fc–FcγR interactions, there are fundamental differences in the activation requirements of specific Fc–mediated effector functions. For CDC activity, stabilization of complement components on the cell surface is essential. This would require a short distance from
epitope to cell membrane. During ADCC, the formation of an immune synapse is essential. This small synapse can only be formed when the NK cells engage antibodies bound in close proximity to the cell membrane, explaining the need for membrane proximal epitopes [175].

**Important epitopes in RSV infection**

Neutralization of RSV is mainly established by antibodies against the RSV F and RSV G protein [177]. Antibodies against the SH and N protein have also been described [178,179] and although these antibodies are not involved in neutralization, they may have other important (Fc-mediated) functions [180]. Capella *et al.* recently showed that antibodies against the pre−F protein were the most prevalent RSV-specific serum antibodies in infants below 2 years of age [181]. Both serum IgG levels against anti-RSV pre−F and G correlated with disease severity in this study.

Various antigenic sites (named Ø and I−VIII) have been described for the two conformational states of the RSV F protein [182,183]. Pre−F-specific antibodies are better neutralizers than post−F-specific antibodies [184]. However, not all pre−fusion F antibodies have similar neutralizing activity [182]. The most potent neutralizing antibodies bind to distal epitopes, suggesting that the neutralizing potential of anti−RSV F antibodies not only relies on the conformation of F on which the epitope is present (e.g. pre−versus post−F), but may also depend on the location of the epitope relative to the viral or cellular membrane. As described above, the proximity to the membrane determines the efficiency of Fc-mediated effector functions [13,174,175]. This suggests that potently−neutralizing antibodies, binding to distal epitopes, may also be efficient inducers of ADCP. Antibodies binding to proximal epitopes are generally less neutralizing, but may be more potent in inducing ADCC and CDC.

The most important antigenic site for the RSV G protein is the central conserved domain (CCD). Despite the high variability of RSV G, antibodies against the CCD are broadly neutralizing against both RSV A and B strains [185]. The G protein CCD binds to the CX3CR1 receptor, leading to attachment of RSV to its target cells [186]. Antibodies against this receptor−binding domain efficiently neutralize RSV infection and decrease pathogenesis by binding soluble G protein, an immune evasion protein secreted by RSV−infected cells [56,57,187]. Soluble G protein has been found to inhibit Fc−mediated antiviral effects of macrophages and complement [75], and
to modulate trafficking of CX3CR1(+) cells [188]. Next to the important roles mentioned above, antibodies against the CCD domain are also able to induce Fc-mediated effector functions like ADCP and ADCC [56].

Taken together, not only the antigen but also the epitope determines the efficacy of antibodies. Interestingly, evidence suggests that targeted epitopes may differ between infants and adults [189], but the effect of these changes on the efficacy of the antibody response is unknown. Further research may uncover the relation between antigenic site and effector functions against RSV infection, and thereby reveal preferred antibody-binding sites for protection against RSV disease.

**FcγR expression and polymorphisms**

Another regulator of Fc-mediated effector functions is the expression pattern and polymorphisms of FcγRs. The majority of leukocytes express more than one FcγR type with varying downstream signaling activities. The level and variety of FcγR expression is tightly regulated during leukocyte development and can be modulated by certain mediators present during infection, inflammation, or even vaccination [103,190]. As stated before, the balance between inhibitory and activating FcγR interactions is crucial in regulating B cell IgG responses [99-101] and skewing APC maturation and antigen presentation [102-105]. Additionally, co-engagement and signaling through other receptors such as TLRs may influence the activation threshold [191]. Altogether, this points out the importance of receptor expression patterns on effector cells.

Besides variation in FcγR expression patterns, single nucleotide polymorphisms (SNPs) in FcγRs occur. Although many SNPs have been identified, only few have been shown to impact receptor function [192]. One of the functional SNPs has been identified in FcγRIIa. Only the R131H allelic variant of this receptor is capable of interacting with IgG2, enabling efficient phagocytosis [193,194]. Another SNP affecting binding affinity has been characterized for FcγRIII, which has two co-dominantly expressed allotypes: V158 and F158. The presence of a valine residue at position 158 increases the affinity for IgG1 and IgG3, augmenting for example NK cell activity [195,196].
**FcγR expression and polymorphisms in RSV infection**

Different FcγRs can have opposing effects on the immune response, as has also been shown for RSV. In FcγR−/− mouse models, Gomez et al. demonstrate that murine FcγRIII (homologue of human FcγRIIA) contributes to viral replication and airway inflammation, whereas murine FcγRIIb (homologue of human FcγRIIb) has a protective effect as was shown by a decrease in viral titers [110]. *In vitro*, RSV infection has been found to increase mFcγRII and mFcγRIII expression in murine macrophage cultures which subsequently showed enhanced phagocytosis [197].

Although the clinical relevance of FcγR SNPs has been studied intensively for auto-immune diseases [198], cancer treatment [199] and various viral infections [200–203], there is no data on the role of these polymorphisms in RSV infection or disease. In a genetic association study, performed to identify genes that are involved in RSV susceptibility, a SNP in FCER1A was found [204]. This polymorphism had previously been found to be associated with altered FcεRI expression levels and allergic disease, supporting the involvement of IgE in RSV-mediated disease.

**IMPLICATIONS FOR VACCINE AND MAB DEVELOPMENT**

Currently there are no market-approved vaccines or antivirals available against RSV. The only available treatment is the administration of a prophylactic F protein–specific mAb (Palivizumab) to reduce hospitalization in high-risk infants [205]. However, the use of Palivizumab is restricted and its cost-effectiveness is often discussed [206]. Improved mAbs with higher efficacy rates are thus highly needed and many research efforts are ongoing to develop these mAbs. A recent clinical trial with a pre-F–specific mAb (Suptavamab) failed to demonstrate efficacy in pre-term infants although the mAb was superior to Palivizumab in neutralization tests *in vitro* and in reducing viral load in the cotton rat model [207] (press release Regeneron, August 14, 2017). The failure of this highly neutralizing mAb indicates that protection against RSV-mediated disease, which is known to be immunopathological in nature, depends on more than just neutralization of the virus.

In addition to efforts made to develop improved therapeutic mAbs, there is an extensive pipeline of vaccines that are currently being tested in different phases of clinical development (https://www.path.org/resources/
The development of vaccines is of great importance, especially for developing countries where RSV-related mortality is high and mAb therapy is inaccessible due to high costs. The majority of vaccine candidates currently in clinical trials are designed to induce systemic IgG, mostly against the RSV F protein. The results of the pre–F–specific SupTuvamab and the recent failures of two F–specific vaccine candidates tested in elderly, imply that a broader and more polyfunctional immune response may be needed to confer protection against RSV–mediated disease [208,209] (press release Novavax, September 15, 2016).

To this date, no accurate correlate of protection has been defined for RSV infection as virus–specific antibody levels or neutralization titers do not seem of use in this respect. The lack of a well–defined correlate of protection complicates the development of new vaccines, as efficacy now has to be demonstrated in expensive large–scale clinical trials. Mounting evidence suggests that antibody effector functions beyond neutralization can contribute to both protection and disease [110,159,180,210].

A balanced activation of different Fc–mediated effector functions is key to prevent excessive inflammation and tissue damage (Figure 9). It will be of importance to implement assays that identify Fc–mediated effector functions of mAbs and vaccine–induced antibodies. Studies in FcγR–knockout mice have indicated the importance of Fc–FcγR interactions for protection against RSV infection [110,180], but the testing of mAbs and vaccines demands high–throughput approaches. Systems serology captures a wide array of antibody characteristics and effector functions. It has proven effective in identifying antibody features that contribute to protection for various (viral) pathogens [19,211,212]. Such an approach will provide detailed information on the characteristics that are required for a protective RSV antibody response.

The ability to generate an antibody profile that selectively binds particular epitopes and FcγRs is important to enable the induction of only the desired antibody effector functions. Recent developments now allow targeted modifications to mAbs that can lead to enhancement or inhibition of specific Fc–mediated antibody effector functions through glyco–engineering or the induction of specific antibody subclasses or isotypes [159]. In the future, this might also be possible for vaccines.

One can conclude from the studies presented above that Fc–FcR
interactions are an integral component of the immune response against RSV and should be considered in the rational design of next generation RSV-specific mAbs and vaccines. Only limited data is available on the effect of specific Fc-mediated antibody effector functions during RSV infection, but it is clear that these can be both beneficial and detrimental for protection against RSV infection and disease outcome. In the future, Fc-mediated effector functions might be harnessed to optimize the efficacy of RSV-specific mAbs and vaccine–induced antibodies. However, our current knowledge on the precise role of individual effector functions in RSV disease is too limited to rationally design such antibodies and vaccines. Therefore, until the individual contributions of Fc-mediated effector functions to protection and disease are unraveled, aiming to induce highly neutralizing antibodies seems the safest approach. These antibodies will

![Diagram](image_url)  

**FIGURE 9** The balance between Fc-mediated protection and enhanced disease.

Antibody effector functions, regulated by differences in antibody characteristics, are suspected to play a role in disease outcome upon RSV infection. Immune activation by Fc-mediated effector functions is likely needed for efficient viral clearance. However, excessive activation may lead to inflammation and tissue damage. A balanced and contained immune response is most likely the key to protection upon infection. **Abbreviations:** Ab, antibody; ADCC, antibody–dependent cell–mediated cytotoxicity; ADCP, antibody–dependent cellular phagocytosis; ADE, antibody–dependent enhancement.
need to halt the infection at the site of entry and thereby prevent excessive (antibody-mediated) immune activation. It remains to be seen whether complete neutralization can be achieved via the induction of serum IgG alone, or whether the induction of mucosal IgA is necessary for reliable neutralization activity. The many clinical trials that are currently ongoing with maternal and neonatal vaccine candidates will show whether these approaches indeed result in protection during the first, most vulnerable, months of life.

CONCLUDING REMARKS

Neutralizing antibody titers do not adequately correlate with protection against RSV disease. Interestingly, antibodies have additional Fc-mediated effector functions besides neutralization, but this area of research is currently underappreciated in the RSV field. With this review, we aim to encourage a paradigm shift from neutralization-based studies towards functional studies examining the precise role of Fc-mediated antibody effector functions in vaccine efficacy and RSV disease. We have evaluated the current literature on the effect of RSV-specific antibodies on NK cells, phagocytes, the complement system, cytokine production and B- and T cell skewing. Multiple in vivo studies using FcγR-knockout mice or modified RSV-specific antibodies indicate the importance of Fc-mediated effector functions in protection from RSV infection and disease [110,159,180,210]. In addition, Fc-mediated effector functions might have a role in ADE of RSV disease [22,23]. However, most studies into vaccine and mAb efficacy still only report antibody (neutralization) titers and disregard any Fc-mediated effector functions. The importance of these antibody effector functions has already been shown for multiple clinically important viral pathogens and is only starting to be explored for RSV. In our view, a better understanding of the broad range of effector mechanisms that are induced by RSV-specific antibodies will greatly contribute to the much-needed development and testing of next generation mAbs and vaccines against this virus.
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PART II

FC-MEDIATED ANTIBODY FUNCTIONALITY IN RSV DISEASE
RSV-SPECIFIC MATERNAL ANTIBODIES ARE LESS POTENT INDUCERS OF NK CELL ACTIVATION IN INFANTS WITH SEVERE RESPIRATORY TRACT INFECTIONS COMPARED TO UNINFECTED CONTROLS

Manuscript in preparation

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ABSTRACT

Background
Respiratory syncytial virus (RSV) is a major cause of severe acute lower respiratory tract infections in infants. Despite extensive research efforts, there is no vaccine available against RSV. In early life, the most important contributors to protection are the innate immune system and maternal antibodies. However, the mechanisms by which these antibodies can protect against severe RSV disease are incompletely understood, as both levels of antibodies as well as neutralization capacity correlate poorly with protection.

Methods
We performed a case–control study including infants hospitalized for RSV infection (n = 43), hernia surgery (n = 16), or RSV-negative viral respiratory tract infections (n = 18). We determined RSV antigen–specific maternal antibody levels in infant plasma using a multiplex immunoassay. Subsequently, we measured the capacity of RSV-specific maternal antibodies to induce natural killer (NK) cell activation, including CD107a surface expression as a measure for antibody–dependent cell–mediated cytotoxicity (ADCC), and interferon gamma (IFN–γ) production.

Results
We found that the concentrations of antigen–specific maternal antibodies did not differ between cases and controls. Interestingly, RSV-specific maternal antibodies, and in particular G-specific antibodies, were able to induce potent NK cell activity in vitro. Finally, RSV-specific maternal antibodies from hospitalized infants with severe lower respiratory tract infections had a significantly lower capacity to induce NK cell IFN–γ production in vitro than antibodies from uninfected controls.

Conclusions
Our results suggest that Fc–mediated antibody functionality contributes to protection against severe RSV disease and warrant further studies to evaluate the potential of harnessing this knowledge to develop an effective vaccine.
INTRODUCTION

Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract disease in young children, with an estimated 118,200 deaths worldwide in children below 5 years of age annually [1]. Despite decades of research, there are currently no market-approved vaccines available against this virus and their development is hampered by the absence of a well-defined correlate of protection. Severe RSV disease is most prevalent in the first six months of life [2, 3], when infants mainly rely on their innate immune system and maternal antibodies for protection against infectious diseases. Considering that maternal vaccination is a potential strategy to prevent RSV disease in infants, it is of pivotal importance to obtain a thorough understanding of the mechanisms by which maternal antibodies might protect against disease, both directly and through their interaction with innate immune cells.

The role of maternal antibodies in RSV infection and disease is unclear. Some studies show that high RSV-specific maternal antibody titers are associated with protection against RSV infection or (severe) disease [4–7]. In contrast, other studies do not show a protective effect [8–11] or even indicate an association between high maternal antibody titers and an increased risk of recurrent wheezing [12]. Strikingly, the vast majority of studies investigating the role of (maternal) antibodies in RSV infection merely look at in vitro binding or neutralization capacity, while additional antibody effector functions are not taken into account.

Natural killer (NK) cells are important innate immune cells in the early response to viral infection and their activity is tightly regulated via their interaction with antigen-specific antibodies [13]. Engagement of the main NK cell Fc gamma receptor (FcγRIIIA) by antibodies bound to virus-infected cells leads to the release of cytotoxic granules containing perforins and granzymes, a process known as antibody-dependent cell-mediated cytotoxicity (ADCC) [14]. In addition, antibody-dependent activation of NK cells is known to result in the secretion of pro-inflammatory cytokines, including interferon gamma (IFN-γ) [15].

Several groups have shown clearance of RSV-infected cells by peripheral blood mononuclear cells (PBMCs) in the presence of monoclonal antibodies, or antibodies from different sources, including breast milk, cord blood, nasopharyngeal secretions, and serum [16–20]. However, none of
these studies shows whether killing was specifically NK cell-mediated. In addition, maternal antibodies from hospitalized RSV patients and controls have never been compared regarding functional properties other than neutralization and antibody-dependent enhancement of infection [21].

Here, we used samples from an observational case–control study of 84 infants (<7 months of age) that were hospitalized for RSV (cases), an inguinal hernia surgery (uninfected controls), or viral respiratory infections other than RSV (RSV-negative infected controls). We first measured plasma levels of antigen–specific maternal IgG against the RSV attachment (G) protein, pre- and post–fusion (F) protein, and nucleoprotein (N) in cases and controls. We then assessed the capacity of plasma containing RSV–specific maternal antibodies to induce NK cell activity in vitro as measured by CD107a surface expression, a marker for degranulation, and IFN–γ production. Finally, we used these assays to investigate differences in the capacity to induce NK cell activity between antibodies from cases and controls. We propose that maternal antibody functionality may be of importance in determining disease outcome.

METHODS

Study design
Plasma samples from hospitalized infants were collected in an observational case–control study in 2010–2014 and have been described before [21]. For the current study, hospitalized infants below 7 months of age with PCR–confirmed RSV infections were included as RSV cases. Age–matched infants admitted for inguinal hernia repair surgery were included as uninfected controls, whereas infants admitted to the hospital for viral respiratory tract infections other than RSV were included as RSV–negative infected controls. The ePlex System (Genmark Dx) was used for identification of respiratory viral pathogens and RSV subtype. No RSV was detected by PCR in nasopharyngeal aspirates from all uninfected controls or RSV-negative infected controls. Blood samples were taken within 24 h after admission. Patients with congenital heart or lung disease, immunodeficiency, or glucocorticoid use were excluded. The study protocols were approved by the Regional Committee on Research Involving Human Subjects Arnhem– Nijmegen (serving as the IRB) and were conducted in accordance with the principles of the Declaration of
Helsinki. Written informed consent was obtained from the parents of all infants.

**Cells and viruses**

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers at the National Institute for Public Health and the Environment (RIVM, the Netherlands). Blood was collected in heparin tubes and the mononuclear fraction was isolated by density gradient centrifugation (Lymphoprep, Nycomed). Isolated cells were cultured in Roswell Park Memorial Institute medium (RPMI, Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin/streptomycin/glutamine (PSG, Gibco), and 5 ng/mL recombinant human IL-15 (Biolegend). Before use, PBMCs were rested overnight at a density of 1x10^6 PBMCs/mL at 37°C and 5% CO₂.

For each of the PBMC donors, the presence of the V158F polymorphism in FcγRIIIA was determined by Sanger sequencing. Genomic DNA was isolated from 1x10^6 PBMCs using DNeasy Blood & Tissue kit (Qiagen) following manufacturer’s protocol. A polymerase chain reaction (PCR) was performed and the PCR product was subsequently sequenced. Primer sequences are detailed in Table 1.

Recombinant RSV–X and RSV–X–ΔG, in which the G gene is replaced by GFP, were propagated in Vero cells as described before [22]. Virus stocks were purified between layers of 10% and 50% sucrose by ultracentrifugation. The 50% tissue culture infectious dose (TCID50) per mL was determined on Vero cells using the Spearman and Karber method [23].

**TABLE 1  Primer sequences**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRIIIA PCR</td>
<td>Forward: 5′-TGGCCACCGTCACCTTATTC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GACACCTCCTAGCTACCCCA-3′</td>
</tr>
<tr>
<td>FcγRIIIA sequencing</td>
<td>Forward: 5′-ATGGCAAGGCGAGGAGTAT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CAAATTCCATGTGATTGC-3′</td>
</tr>
</tbody>
</table>
Control sera
The International Standard for Antiserum to Respiratory Syncytial Virus (16/284, National Institute for Biological Standards and Control) was used as control in the NK cell activation assay. An in-house reference serum pool (RIVM) known to have high RSV titers was used as standard in the multiplex immunoassay. Additionally, cord blood (CB) plasma pooled from >10 individual donors was used as a standard. RSV-infected Vero cells were used to deplete all RSV-specific antibodies from the CB pool, whereas RSV-ΔG-infected Vero cells were used to deplete all RSV-specific antibodies except those specific for RSV G. Mock-depleted CB control was made by incubation with uninfected Vero cells. Depletion of RSV-specific antibodies was confirmed by multiplex immunoassay as described below. Cord blood samples were collected from umbilical cords of healthy neonates born by cesarean delivery at Radboudumc Nijmegen (the Netherlands). All mothers provided written informed consent.

RSV-specific multiplex immunoassay (MIA)
To quantify the concentration of RSV-specific IgG and IgA, a multiplex immunoassay was performed as described before for IgG [24]. Briefly, plasma samples were diluted 200 and 4000 times and incubated with RSV antigen-coupled beads, including G_{A'}, G_{B'}, pre-F (DS–CAV1 form), post-F, and N. Except for N, which was produced in Escherichia coli, all antigens were produced in eukaryotic cells. The bound antibodies were detected with secondary R-phycoerthrin labeled goat anti-human IgA F(ab')_{2} (Southern Biotech) or goat anti-human IgG F(ab')_{2} (Jackson Immunoresearch Laboratories). Samples were measured on a Bio–Plex 200 (Luminex Corporation) in combination with Bio–Plex Manager software version 6.1 (Bio–Rad). Later in the study the Bio–Plex 200 was replaced by a Flexmap 3D (Luminex Corporation) in combination with Xponent version 4.2 (Luminex Corporation) and Bio–Plex Manager Software version 6.1. For each analyte, median fluorescence intensity (MFI) was converted to arbitrary units/ml (AU/ml) by interpolation from a 5–parameter logistic standard curve from an in–house reference serum pool known to have high RSV antibody concentrations [24]. Although all plasma samples were tested in the NK cell activation assay, not enough volume was left for some of the samples to be tested in the IgG (n = 8) and/or IgA (n = 11) MIA. These samples were divided over the 3 different groups.
**NK cell activation assay**

Sterile Immulon ELISA plates (ThermoScientific) were coated with intact RSV particles at a concentration of $1.7 \times 10^5$ TCID50/well. After overnight coating at 4°C, wells were washed with PBS and blocked with 10% FCS in PBS for 30 min at RT. After washing with PBS, coated plates were incubated for 2 hours at 37°C with 10-fold diluted plasma, to allow for antigen-specific immune complex formation. Subsequently, unbound antibodies and other plasma constituents were washed away and $1 \times 10^6$ PBMCs were added per well, together with Brefeldin A (BD Bioscience), and anti-human CD107a–PerCP/Cy5.5 (clone H4A3; BioLegend). PBMCs were incubated with the opsonized virions for 4 hours at 37°C, after which the cells were stained for flow cytometric analysis as described below. Incubation of PBMCs in RSV-coated plates in the absence of antibodies was used to determine the background signal. The PBMC supernatants were stored at −80°C and used for detection of perforin secretion as described below.

Due to the limited amount of fresh PBMCs per donor, we were unable to test all samples using cells of all 6 donors. For this reason, we could test 63 of the 84 plasma samples on cells from all 6 PBMC donors. For 18 samples, we were able to test with PBMCs from 2–5 different donors. Three samples were only tested on a single donor; these were all RSV-negative infected controls. Excluding these 3 samples had no effect on the subsequent statistical analysis. The mock-depleted CB pool was used for normalization between the different PBMC donors.

**Flow cytometric analysis**

PBMCs that were incubated with opsonized virions as described above were stained for flow cytometric analysis. Extracellular staining was performed with anti-human CD3–FITC (clone UCHT1, Biolegend), anti-human CD56–PE (clone HCD56, Biolegend), and Fixable Viability Staining–eFluor780 (eBioscience). After fixation and permeabilization, cells were intracellularly stained with anti-human IFN-γ–PECy7 (clone B27, Biolegend). Flow cytometric analysis was performed using the FACS LSR Fortessa X20 (BD Bioscience). In all experiments, NK cells were gated as the CD3–, CD56+ population. The gating strategy is depicted in Supplementary Figure 1. FlowJo software V10 (FlowJo, LLC) was used for data analysis.
Perforin ELISA
The supernatants collected after the NK cell activation assay were used to determine the total perforin secretion by ELISA (Mabtech). The ELISA was performed following the manufacturer’s protocol and optical density was measured at 450 nm using an ELISA reader (BioTek).

Statistical analysis
Comparison of two groups or data points was performed by using a nonparametric Mann-Whitney test. Multiple comparisons were analyzed by using a nonparametric Kruskal-Wallis test, followed by Dunn’s multiple comparisons test. Multiple comparisons of categorical values were tested by Chi-square test. $P$ values <0.05 were considered statistically significant. Statistical analyses were performed with Prism 8 software (GraphPad).

RESULTS

Clinical characteristics of study subjects
Plasma samples were obtained from a total number of 84 infants below 7 months of age that were hospitalized for RSV infection (cases), hernia surgery (uninfected controls), or viral respiratory infections other than RSV (RSV-negative infected controls). To ensure that our analyses were restricted to maternal antibodies, all plasma samples were screened for the presence of RSV-specific IgA. Since this antibody isotype is not transplacentally transferred and breastmilk IgA does not reach the blood, its presence in plasma is indicative of a humoral response by the child itself due to previous exposure. Based on the results, we excluded those children (n = 5) that tested positive for IgA (AU/mL > 0.2) against a minimum of 3 RSV antigens (Figure 1A). Excluded samples are indicated with white squares. Asterisks indicate the concentrations found in the in-house reference serum pool from adults, known to contain RSV-specific IgA. In addition, we excluded 2 children that had presumably received Palivizumab, based on the fact that they displayed unusually high IgG levels for both pre-F and post-F, while levels for N and G were low or even below detection level (data not shown).

The clinical and demographic characteristics of the children included in further analyses are depicted in Table 2. There was no significant difference in age, gestational age or breastfeeding between the three groups. However,
there was a significant difference in division of sex between the three groups (P = 0.0459). A higher percentage of infants was infected with RSV-A (70%) compared to RSV-B (30%). The group of RSV patients contained both RSV mono-infections (n = 28) and co-infections (n = 15) with other respiratory viral pathogens, with rhinovirus being the most prevalent.

**FIGURE 1** Characterization of the infant (maternal) RSV-specific antibody repertoire.

An RSV-specific multiplex immunoassay was performed to quantify the concentration of IgA and IgG specific for G, pre-F, post-F, and N in plasma. **A.** Relative concentration of plasma IgA depicted for each of the RSV antigens. Children that tested positive for IgA against a minimum of 3 RSV antigens (n = 5) are excluded for subsequent analyses and indicated with white squares. Pink asterisks indicate the IgA levels of the in-house RSV reference serum pool. **B.** Relative concentration of plasma IgG depicted for each of the RSV antigens. Graphs depict geometric mean and SD. **C.** Relative concentration of RSV-specific plasma IgG plotted against age. **D.** Correlation analysis of RSV N- and RSV pre-F-specific maternal antibody levels. Nonparametric Spearman correlation analysis was used to assess correlations. **Abbreviations:** AU, arbitrary units; IgA, immunoglobulin A; IgG, immunoglobulin G; RSV, respiratory syncytial virus.
TABLE 2  Demographic and clinical characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>RSV cases (n = 43)</th>
<th>Uninfected controls (n = 16)</th>
<th>RSV-negative controls (n = 18)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in days (range)</td>
<td>70 (11–185)</td>
<td>79 (28–175)</td>
<td>89 (16–202)</td>
<td>0.3260b</td>
</tr>
<tr>
<td>Gestational age in weeks (range)</td>
<td>38 (34–41)</td>
<td>NA</td>
<td>38 (35–41)</td>
<td>0.9749d</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>22/21</td>
<td>12/4</td>
<td>5/11a</td>
<td>0.0459f</td>
</tr>
<tr>
<td>Breastfeeding (%)</td>
<td>21 (50%)</td>
<td>NA</td>
<td>7 (39%)</td>
<td>0.8246f</td>
</tr>
<tr>
<td>RSV types</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>30 (70%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>13 (30%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Co-infections (%)</td>
<td>15 (35%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

\[a\] Age of one infant was not recorded, \[b\] Comparisons between RSV patients, uninfected controls, and RSV-negative controls; Kruskal–Wallis test followed by Dunn’s test to adjust for multiple comparisons, \[c\] Gestational age of 1 RSV patient, all uninfected controls, and 3 RSV-negative controls was not recorded, \[d\] Comparisons between RSV patients, uninfected controls, and RSV-negative controls; Mann–Whitney test, \[e\] Sex of two infants was not recorded, \[f\] Comparisons between RSV patients, uninfected controls (only for sex), and RSV-negative controls; Chi-square test, \[g\] Breastfeeding was not recorded for 1 RSV patient, all the uninfected controls, and 3 RSV-negative controls.

The concentration of RSV-specific maternal IgG does not differ between RSV cases and controls

We used a multiplex immunoassay (MIA) to quantify the concentration of maternal IgG specific for the RSV attachment (G) protein, fusion (F) protein, and nucleoprotein (N). G protein from both an A- and a B-strain was included, as these sequences are highly variable between the two RSV subtypes [25]. Both the pre- and post-fusion conformations of the F protein were included, as these harbor partly overlapping but also distinct epitopes [26].
As has been published by others [7,24], the concentration of RSV G–specific antibodies was low compared to antibodies against the RSV F protein (Figure 1B). In addition, the concentration of N–specific antibodies was high. Although the N protein is generally regarded as a viral core protein, it has also been described to be present on the surface of viral particles and RSV-infected cells [27], which could explain the high levels of N–specific antibodies. Despite the cross-sectional design of the study, we could observe a gradual waning of maternal antibody concentrations with increasing age (Figure 1C). As would be expected, there was a strong correlation between the concentrations of antibodies targeting different RSV antigens, as exemplified for pre-F and N (Figure 1D). To assess a possible relation with RSV disease, we compared the concentrations of RSV antigen–specific antibodies between RSV-infected cases, uninfected controls, and RSV-negative infected controls. The concentrations of antigen–specific antibodies did not significantly differ between the three groups (Figure 2A–E), confirming previous findings in a subset of this cohort [11]. Taken together, these findings indicate that all children possess RSV–specific maternal antibodies, but their concentration does not explain susceptibility to severe RSV disease.

**RSV–specific maternal antibodies induce NK cell activity in vitro**

We then set out to explore possible differences in NK cell activation by maternal RSV–specific antibodies, as NK cells play an important role in the control of viral lung infections. We first assessed whether maternal RSV–specific antibodies are capable of inducing NK cell activity in vitro. Incubation of primary PBMCs with RSV–antibody complexes formed with a dilution series of a mock–depleted cord blood plasma pool, naturally containing RSV–specific maternal antibodies, showed a concentration–dependent activation of NK cells as measured by CD107a surface expression (Figure 3A). Surface expression of CD107a is an established marker for NK cell degranulation [28]. A pool of cord blood plasma largely lacking RSV–specific antibodies (RSV–depleted) showed strongly reduced NK cell activation, whereas cord blood plasma containing almost exclusively RSV G–specific antibodies (RSV–ΔG–depleted) induced similar activation as mock–depleted plasma. In addition to the release of cytotoxic granules, NK cells are potent producers of IFN–γ. We therefore also performed intracellular staining for this cytokine, which gave comparable results to
FIGURE 2 The RSV–specific antibody composition does not differ between uninfected controls, RSV patients, and RSV–negative infected controls.

An RSV–specific multiplex immunoassay was performed to quantify the amount of IgG specific for G, pre–F, post–F, and N in plasma. A–E. Comparison of the amount of antibodies against RSV–A attachment protein G (A), RSV–B attachment protein G (B), pre–fusion F protein (C), post–fusion F protein (D), and nucleoprotein N (E) between uninfected controls, hospitalized RSV patients, and hospitalized RSV–negative infected controls. All graphs depict geometric mean and SD. Kruskal–Wallis test with Dunn’s multiple comparisons test was used for comparison between multiple groups. Abbreviations: AU, arbitrary units; IgG, immunoglobulin G; RSV, respiratory syncytial virus.
FIGURE 3  RSV–specific maternal antibodies induce NK cell activity.
A–B. NK cell–specific CD107a surface expression (A) and IFN–γ production (B) of three different PBMC donors after 4 hour incubation with viral particles opsonized with mock–depleted cord blood, RSV–depleted cord blood, or RSV–ΔG–depleted cord blood. Graphs depict geometric mean and SD. Kruskal–Wallis test with Dunn’s multiple comparisons test was used for comparison between the differently depleted cord blood pools of the same dilution (*p < 0.05, **p < 0.01).
C–D. RSV–specific multiplex immunoassay of mock–depleted cord blood (complete bar) compared to RSV–depleted cord blood (filled bar, C), or RSV–ΔG–depleted cord blood (filled bar, D). Abbreviations: AU, arbitrary units; CB, cord blood; IFN–γ, interferon gamma; NK cells, natural killer cells; RSV, respiratory syncytial virus.
CD107a surface expression (Figure 3B).

To confirm the depletion of RSV–specific antibodies, we assessed their concentration in the various cord blood plasma pools using the RSV–MIA. Upon RSV–depletion, we observed a strong decrease in all measured specificities compared to the mock–depleted cord blood pool (Figure 3C). The RSV–ΔG–depleted cord blood plasma pool showed comparable G–specific antibody levels as the mock–depleted pool, while the concentrations of the other measured specificities were even lower than upon RSV–depletion (Figure 3D). Interestingly, the relatively high level of NK cell activation by RSV–ΔG–depleted cord blood plasma, containing similar amounts of G–specific antibodies as mock–depleted cord blood plasma but lower amounts of F– and N–specific antibodies than RSV–depleted cord blood, suggests that the majority of antibody–mediated NK cell activation is induced by RSV G–specific antibodies (Figure 3A–B). Taken together, these results show that RSV–specific maternal antibodies can induce NK cell activity. As described before, RSV G–specific antibodies seem to be the most potent inducers of this activity [19].

Effect of FcγRIIIA polymorphism on NK cell activation

Notably, we observed considerable variation in CD107a surface expression and IFN–γ production between PBMC donors, prompting us to explore the underlying cause and assess the possibility for normalization. The presence of a common single nucleotide polymorphism (SNP) in FcγRIIIA, replacing the phenylalanine at position 158 with a valine residue (V158F), has been described to influence NK cell ADCC activity [29,30]. We therefore genotyped 6 PBMC donors: 4 were heterozygous (F/V), 1 was homozygous for valine (V/V), and 1 was homozygous for phenylalanine (F/F).

Using a dilution series of the WHO RSV reference serum, we assessed RSV–specific antibody–mediated NK cell activation in the genotyped donors. CD107a surface expression was significantly enhanced on NK cells from heterozygous donors compared to the homozygous donors (Figure 4A). Looking at IFN–γ production, a low response was not only confined to homozygous donors, as one heterozygous donor also showed low IFN–γ production (Figure 4B). To correct for these donor differences in subsequent assays, a mock–depleted cord blood plasma sample was included and used for normalization. After normalization, there was no difference in antibody–mediated NK cell activation between donors (Figure 4C–D).
**FIGURE 4** Influence of FcyRIIIA polymorphism on NK cell activation.

**A–B.** NK cell–specific CD107a surface expression (A) and IFN–γ production (B) of six different PBMC donors after 4 hour incubation with viral particles opsonized with the indicated concentrations WHO RSV reference serum. **C–D.** CD107a surface expression (C) and IFN–γ production (D) after normalization to the mock–depleted cord blood pool. All graphs depict geometric mean and SD. Mann–Whitney test was used for comparison between the average of heterozygous donors and the average of homozygous donors at a concentration of 500 IU/ml (**p < 0.01). **Abbreviations:** AU, arbitrary units; FcyRIIIA, Fc gamma receptor IIIA; IFN–γ, interferon gamma; NK cells, natural killer cells; RSV, respiratory syncytial virus.
Maternal RSV-specific antibodies from patients with severe respiratory tract infections induce less potent NK cell activity compared to uninfected controls

To evaluate whether RSV-specific maternal antibody functionality, other than neutralization, relates to disease severity, we finally assessed the capacity of these antibodies from RSV patients and controls to induce activation of primary NK cells from healthy donors. We found considerable variation in the capacity of plasma containing maternal RSV-specific antibodies to induce CD107a surface expression (Figure 5A). Furthermore, RSV-specific antibodies from both RSV patients and RSV-negative infected controls induced slightly less CD107a surface expression than those from uninfected controls, but this difference was not statistically significant. Interestingly, we found that RSV-specific antibodies from both RSV patients and RSV-negative infected controls did show a significantly decreased potential to induce IFN-γ production compared to antibodies from uninfected controls (Figure 5B).

CD107a surface expression is used as a proxy for NK cell degranulation. To assess the correlation between CD107a surface expression and degranulation, we measured perforin secretion in the supernatant of the NK cell activation assay for one PBMC donor. We observed a strong positive correlation between NK cell CD107a surface expression and perforin concentration (Figure 5C).

Finally, since the capacity of antibodies to induce NK cell activity could be directly related to antibody levels, we assessed the correlation between RSV antigen–specific antibody levels and IFN-γ production. As exemplified by the correlation between pre-F–specific antibody concentrations and IFN-γ production (Figure 5D), there was only a moderate correlation between the antibody levels and NK cell activation, suggesting that other factors besides concentration contribute to the functional capacity of these antibodies. In summary, these findings indicate that, in contrast to RSV-specific maternal antibody concentrations, the capacity of these antibodies to induce IFN-γ production by NK cells is significantly decreased in hospitalized infants with severe respiratory tract infections compared to uninfected controls.
FIGURE 5  NK cell activation in RSV patients compared to controls.

A–B. Normalized CD107a expression (A) and IFN-γ production (B) after 4 hour incubation of PBMCs with viral particles opsonized with 10-fold diluted plasma samples from uninfected controls, RSV patients, or RSV-negative infected controls. Graphs depict geometric mean and SD. Kruskal–Wallis test with Dunn’s multiple comparisons test was used for comparison between multiple groups (*p < 0.05, **p < 0.01). C. Correlation analysis between perforin secretion in the supernatant and CD107a surface expression of one donor. D. Correlation analysis between RSV pre-F-specific antibody levels and IFN-γ production. Uninfected controls are indicated in black, RSV patients in grey, and RSV-negative infected controls in white. Nonparametric Spearman correlation analysis was used to assess correlations. Abbreviations: IFN-γ, interferon gamma; NK cells, natural killer cells; RSV, respiratory syncytial virus.
PART II

DISCUSSION

Studies investigating the role of (maternal) antibodies in RSV infection mostly focus on neutralization and binding titers. Whereas antibody-mediated protection against RSV disease in vivo may depend at least in part on direct neutralization, additional antibody functionalities are likely to be important contributors. Interestingly, there is increasing evidence that Fc-mediated antibody functionality plays an important role during RSV infections (reviewed in [31,32]). In two independent in vivo studies, modification of the Fc domain of RSV-specific antibodies has shown major effects on lung viral titer [33,34]. These results strongly support the idea that Fc-dependent mechanisms may significantly enhance the efficacy of RSV-specific antibodies. In the present study, we measured RSV antigen-specific antibody levels, but these concentrations did not explain susceptibility to severe RSV disease. In addition, we analyzed the capacity of RSV-specific maternal antibodies to induce NK cell activity. We demonstrated that these antibodies, and in particular those targeting the G protein, can induce CD107a surface expression and IFN-γ production in primary NK cells. Importantly, we found that RSV-specific maternal antibodies from RSV patients and RSV-negative infected controls less potently induce IFN-γ production compared to uninfected age-matched controls.

Antibody-mediated NK cell activation has gained increasing attention for its role in multiple other infectious diseases. The capacity of antibodies to induce NK cell ADCC correlated with the control of mycobacterium tuberculosis [35] and the killing of chlamydia trachomatis [36]. For HIV, ADCC-inducing antibodies have been identified as a key correlate of protection in the RV144 HIV vaccine trial [37–39]. For influenza, the role of ADCC was shown to be protective in some studies [40,41], but others point to the involvement of ADCC in exaggeration of the immune response [42–44]. Taken together, these examples show that the capacity of antibodies to induce ADCC can significantly contribute to protection.

In our study, the difference in antibody functionality between patients with severe lower respiratory tract infections and uninfected controls did not seem to be explained by antibody concentrations alone, thus, other factors are likely to play a role. It remains to be determined which factors are the most important regulators of the functionality of RSV-specific maternal
antibodies. One factor that affects antibody functionality is antigen-specificity. Despite the difference in experimental set-up, we were able to corroborate previous findings from Gupta et al., who showed that RSV G–specific antibodies have an increased capacity to mediate clearance of RSV-infected cells compared to total intravenous immunoglobulin or a monoclonal antibody against RSV F [19]. In addition to antigen-specificity, several other antibody characteristics are known to influence antibody functionality. First, the epitope specificity of antibodies determines which effector functions are induced, as antibodies that bind membrane-proximal epitopes mediate ADCC more efficiently than distal binders [45]. Second, glycosylation of the Fc domain was shown to be an important regulator of antibody functionality. Specifically, the absence of the core fucose on the Fc glycan enhances the interaction with FcγRIIIA, thereby increasing ADCC activity [46]. Finally, different IgG subclasses have varying capacities to induce Fc-mediated effector functions [47].

Besides antibody characteristics, FcγR polymorphisms and expression patterns on effector cells can affect Fc-mediated antibody functions. We observed decreased CD107a surface expression of both homozygous (V/V and F/F) donors compared to the heterozygous (V/F) donors. It has been described that the presence of a valine residue at position 158 in FcγRIIIA results in increased binding affinity for IgG1 and IgG3 [29,30]. However, in the presence of two valine residues, resulting in the high affinity V/V phenotype, NK cells are pre-loaded with a high amount of FcγRIIIA-bound IgG [30], which could potentially interfere with the binding of RSV-specific antibodies in our assay. We did not assess the expression of FcγRIIB or FcγRIIC on the primary NK cells used in our experiments. Expression of FcγRIIB is seen in some individuals and effectively inhibits ADCC activity [48], whereas expression of FcγRIIC can both inhibit or stimulate ADCC activity, depending on the isoform that is expressed [49]. Although the effect of NK cell FcγR polymorphisms on IFN-γ production has never been assessed, our results suggest that other factors besides the V158F polymorphism play a role.

The main limitation of our study is the fact that children were not sampled at a time point before infection, but upon admission to the hospital. Due to the lack of this pre-infection sample, we were unable to investigate whether the low antibody functionality was a cause or consequence of severe respiratory tract disease. It has been shown that both inflammation
and infection can affect antibody functionality [50]. This could explain the significantly lower induction of IFN-\(\gamma\) production in both groups with severe lower respiratory tract infections, independent of the etiologic agent. Alternatively, antibodies with lower functionality could have already been present before infection, leading to disease susceptibility due to a reduced capacity to activate NK cells. Future research, preferably using samples obtained before infection, is needed to address this question. Another limitation is the lack of information on whether the uninfected controls will develop severe disease after encountering their first RSV infection. However, considering that virtually every child is infected by the age of two and only 2-3% of RSV-infected infants is admitted to the hospital [2,4], we believe the uninfected control group can be considered representative for infants that will experience a mild RSV infection.

To date, no correlate of protection has been defined for RSV infection. The lack of a well-defined correlate of protection complicates the development of new vaccines, as efficacy has to be demonstrated in large-scale clinical trials. In the current study, NK cell activation was highly variable within the groups. This indicates that the capacity of maternal antibodies to activate NK cells may partly explain RSV hospitalization but it is highly unlikely to be the only determinant, indicating that additional antibody characteristics and functionalities should be investigated. The use of a systems serology approach in the RSV research field may offer an unbiased and comprehensive way to systematically investigate antibody characteristics and effector functions. It has already proven effective in identifying antibody features that contribute to protection against various (viral) pathogens [35,51,52]. Also for RSV, such a systems serology approach may provide a useful framework to evaluate new vaccines and monoclonal antibodies.

In conclusion, our results highlight the need for further investigations into the role of Fc-mediated antibody effector functions in protection against RSV disease. In addition, our findings provide additional incentive for evaluating the RSV G protein as a vaccine target, where most efforts are currently focused on the RSV F protein. A thorough understanding of the protective capacity, in its broadest sense, of maternal RSV-specific antibodies will be invaluable for the development of a safe and effective vaccine against this devastating pathogen.
REFERENCES


IN VITRO ENHANCEMENT OF RESPIRATORY SYNCYTIAL VIRUS INFECTION BY MATERNAL ANTIBODIES DOES NOT EXPLAIN DISEASE SEVERITY IN INFANTS

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ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of severe respiratory illness in infants. At this young age, infants typically depend on maternally transferred antibodies (matAbs) and their innate immune system for protection against infections. RSV–specific matAbs are thought to protect from severe illness, yet severe RSV disease occurs mainly below 6 months of age, when neutralizing matAb levels are present. To investigate this discrepancy, we asked if disease severity is related to antibody properties other than neutralization. Some antibody effector functions are mediated via their Fc binding region. However, it has been shown that this binding may lead to antibody–dependent enhancement (ADE) of infection or reduction of neutralization, both possibly leading to more disease. In this study, we first showed that high levels of ADE of RSV infection occur in monocytic THP-1 cells in the presence of RSV antibodies and that neutralization by these antibodies was reduced in Vero cells when they were transduced with Fc gamma receptors. We then demonstrated that antibodies from cotton rats with formalin–inactivated– (FI–) RSV–induced pulmonary pathology were capable of causing ADE. Human matAbs also caused ADE and were less neutralizing in vitro in cells that carry Fc receptors. However, these effects were unrelated to disease severity because they were seen both in uninfected controls and in infants hospitalized with different levels of RSV disease severity. We conclude that ADE and reduction of neutralization are unlikely to be involved in RSV disease in infants with neutralizing matAbs.

Importance

It is unclear why severity of RSV disease peaks at the age when infants have neutralizing levels of maternal antibodies. Additionally, the exact reason for FI–RSV–induced enhanced disease, as seen in the 1960’s vaccine trials, is still unclear. We hypothesized that antibodies present in either of these conditions could contribute to disease severity. Antibodies can have effects that may lead to more disease instead of protection. We investigated two of those effects: antibody–dependent enhancement of infection (ADE) and neutralization reduction. We show that ADE occurs in vitro with antibodies from FI–RSV–immunized RSV–infected cotton rats. Moreover, passively acquired maternal antibodies from infants had the capacity to induce ADE and reduction of neutralization. However, no clear association with disease severity was seen, ruling out that these properties explain disease in the presence of maternal antibodies. Our data contribute to a better understanding of the impact of antibodies on RSV disease in infants.
INTRODUCTION

Human respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract disease in young children [1]. Hospitalization for severe RSV–mediated disease is most frequent between six weeks and six months of life [2,3]. It seems widely accepted that RSV–neutralizing, transplacentally transferred maternal antibodies (matAbs) can lower the risk for RSV infections [1,4,5]; however, many studies fail to reproduce these results [6–8]. There is no consensus on how much antibodies are sufficient for protection, but an average concentration of maternal antibodies seems insufficient.

Maternal antibody levels are high during the first six months after birth. Strikingly, severe RSV disease most frequently occurs in this period of life [2,3], indicating RSV can infect infants even though matAbs are present. For these children who become infected with RSV, the role of matAbs in RSV disease is unclear. Associations between the severity of symptoms and classical serological parameters such as RSV neutralization titer or RSV–specific antibody levels have not been observed so far [4,6]. Yet, antibodies have additional effector functions, generally mediated via their Fc binding region, that contribute to immune defense. In this study, we investigate whether consequences of antibody–Fc receptor interactions could be related to the severity of symptoms during RSV infection.

Antibody–dependent enhancement (ADE) of RSV infection has been demonstrated in vitro [9] in monocytic cell lines. ADE means that RSV–specific antibodies in human serum, as well as monoclonal antibodies, increase the number of RSV–infected cells, when those cells carry Fc gamma receptors (FCGR) [10,11]. FCGR–carrying leukocytes, which are present in the lungs or recruited during infection, might be more readily infected and activated by RSV–antibody complexes compared to RSV alone. In addition to causing ADE, Fc receptors lower the RSV neutralization capacity of most monoclonal antibodies [12], allowing RSV to infect even in the presence of otherwise neutralizing antibody titers. Whether FCGR binding influences RSV (immuno–) pathology and severity of RSV disease has not been studied so far, but the ongoing pursuit of RSV vaccines demands more knowledge about the role of antibodies in RSV disease. Therefore, we investigated whether reduced neutralization and enhanced RSV infection of FCGR–bearing cells by maternal antibodies
that should provide passively acquired immunity relates to clinical disease severity in infants. Moreover, antibodies generated as part of active immunity induced by formalin-inactivated- (FI-) RSV vaccination have been shown to contribute to enhanced pathology [13]. Therefore, we also investigated whether reduced neutralization and enhanced RSV infection of FCGR-bearing cells relates to pathology in the FI-RSV cotton rat model.

We assessed two FCGR-mediated effects of RSV-specific antibodies: antibody-dependent enhancement of infection in monocytic THP-1 cells, and reduction of neutralization capacity in cells transduced with Fc gamma receptors. Both were investigated for passively transferred matAbs by titration series of plasma from human cord blood and for actively acquired antibodies from immunized cotton rats, a small animal model that is highly susceptible to RSV and shows vaccine-enhanced pulmonary pathology [14,15]. To assess possible associations with disease severity, plasma samples from infants with acute primary RSV infection were tested as well as sera from cotton rats showing FI-RSV-induced enhancement of pathology.

MATERIALS AND METHODS

Study design
Plasma samples from healthy controls and hospitalized infants with RSV infections used in this study, have been described before [6]. Hospitalized children below 1 year of age with PCR-confirmed RSV infections were included during 2011–2013. In the previous study only samples below 3 months of age were included [6]. In this study we included all 51 children, of which 20% was > 3 months of age. Blood samples were taken within 24 h after admission. Patients with congenital heart or lung disease, immunodeficiency or glucocorticoid use and infants born at a gestational age below 35 weeks were excluded. For analysis, patients were classified with severe symptoms when they required mechanical ventilation and admission to the intensive care unit. Infants with moderate disease were only monitored or received oxygen therapy. Infants below 1 year of age requiring surgery for an inguinal hernia repair were included as healthy controls. Nasopharyngeal aspirates from all uninfected individuals were RSV negative. Gender, gestational age, presence of breastfeeding and presence of parental smoking were comparable between all groups. The
study protocols were approved by the Regional Committee on Research involving Human Subjects Arnhem–Nijmegen (serving as the IRB) and were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the parents of all infants. A potential limitation of our study is the difficulty to separate the presence of matAbs from endogenous RSV–induced antibodies of the infant. However, with a median onset of disease of three days and the short time between symptoms and hospitalization, production of endogenous RSV–IgG is unlikely to have reached significant levels at the time of sampling. About 80% of patients were below 3 months of age. As Dutch infants in general suffer their first respiratory infection after 3 to 7 months of age, infants in our study most likely experienced a primary RSV infection [16]. Therefore, it is plausible that we detect mainly matAbs [17].

**Cells**

Monkey kidney epithelial Vero–CCL81 cells were propagated in DMEM supplemented with 5% fetal calf serum (FCS) and 1% penicillin/streptomycin/glutamax (PSG). The Vero cell–derived cell line expressing FCGR2a (Vero2a) was described earlier [12] and FCGR2a–expression was confirmed by flow cytometry (data not shown). Monocytic THP-1 cells were cultured in RPMI supplemented with 10% FCS and PSG [18]. Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers. Blood was collected in heparin tubes and the PBMC fraction was isolated by density gradient centrifugation using Lymphoprep (Nycomed).

**Virus and vaccine preparation**

Live–attenuated recombinant RSV–X (rRSV) [19], recombinant RSV–X containing a GFP gene (designated RSV [19]) and RSV–A2, were propagated in Vero cells as described before [19] (Dutch GMO license IG–99–210). Virus stocks were purified in between layers of 10% and 50% sucrose by ultracentrifugation. FI–RSV and formalin–inactivated cell culture supernatant was prepared from RSV–A2 as described previously [20].

**Cotton rats**

Cotton rats (*Sigmodon hispidus*) were held at the animal facilities of Intravacc (Netherlands) and experiments were approved by the Animal Ethical Committee of RIVM. Animals were immunized intranasally with
10^5 infectious units rRSV or uninfected Vero cell supernatant (mock) or intramuscularly with a high or low dose Fl-RSV (1:5 and 1125 diluted in PBS, respectively) on day 0 and 21. Animals were challenged intranasally with 10^5 infectious units RSV-A2 on day 49. Animals were sacrificed, blood plasma was collected, and lungs fixed on day 53. Histopathologic analysis of lung sections using haematoxilin and eosin staining was done by Paul Roholl (Microscope Consultancy, Netherlands) as described previously [21]. To prepare single cell suspensions, lung tissue of untreated animals was separated after DNase/collagenase digestion. Isolated lung cells were phenotyped by staining with antibodies to an epithelial marker, pan-cytokeratin [22], and T cell marker CD3ε [23].

**RSV infection and serology**

THP-1 cells, human PBMCs or cotton rat lung cells were used to measure ADE. Immune complexes were formed by pre-incubation of RSV with antibodies for 1 h at 37°C. Cells were inoculated by spinoculation for 1 h at 700 × g at 20°C in duplicates. Next, cells were washed with PBS and replenished with culture medium. Overnight incubation at 37°C was followed by flow cytometric analysis of GFP fluorescence using a FACS Canto II (BD Biosciences). RSV neutralization assays were carried out on Vero and Vero2a cells. Assays were performed in triplicate as described earlier [19]. 50% plaque reduction neutralization titers (PRNT50) were calculated from non-linear regression using Prism 7 (GraphPad) after normalization to antibody–untreated, RSV–infected samples. Spinoculation had no effect on ADE or change of PRNT50s in any of the infection assays (data not shown). Antibody–binding sites of FCGRs were blocked by adding 3 µg/mL staphylococcal FLIPr–like protein (kind gift from Kok van Kessel, University Medical Center Utrecht) [24] to THP–1 cells during RSV neutralization assays. Cells were pretreated for 1 h and supplemented during infection and initial incubation steps. FLIPr–like protein was removed with the inoculum.

RSV–specific IgG concentrations in plasma samples were determined by ELISA against RSV particles as described previously [6]. 1 mg/ml IVIg (KIOVIG, Baxter) was referred to as 1 arbitrary unit.
RESULTS

Antibody-dependent enhancement (ADE) of RSV infection and reduction of RSV neutralization (ΔPRNT50)

Infants that encounter RSV in the first months of life have maternal antibodies. Despite the presence of maternal antibodies, a subset of infants that encounters RSV becomes severely ill. The antibodies of severely diseased infants are not neutralizing enough to prevent infection, but they can induce multiple other effector functions through FCGR interactions. In this paper, we investigate the impact of these Fc receptor-mediated functions of maternal antibodies on the severity of disease symptoms during RSV infection. We assessed two antibody-Fc receptor mediated effects \textit{in vitro}: antibody-dependent enhancement (ADE) and reduction of RSV neutralization.

ADE of RSV infection in the presence of human serum has previously been demonstrated in monocytic cell lines, which carry Fc receptors \[10,11\]. ADE of RSV infection can be induced by sub-neutralizing concentrations of antibodies. At low concentrations, antibodies are not able to neutralize infection, but bind RSV and mediate binding to Fc receptors. This attachment and subsequent internalization facilitates higher infection rates than in the absence of antibodies, and is schematically illustrated for monocytic THP-1 cells in \textbf{Figure 1A}.

The second effect we investigated is the ability of the FCGR to reduce the neutralization capacity (PRNT50) of antibodies. Antibodies have higher neutralization potential on cells lacking FCGR compared to the same cell type expressing FCGR as is shown for wildtype Vero cells compared to Vero cells expressing FCGR \[12\]. This change in neutralization mediated by FCGR, we called ΔPRNT50 throughout the manuscript and is illustrated in \textbf{Figure 1B}. ΔPRNT50 represents the factor of reduction of neutralization and is calculated by dividing the PRNT50 in Vero cells by the PRNT50 in Vero cells expressing FCGR2a (Vero2a).
ADE in primary cells and monocytic cell line THP-1

ADE of RSV infection in the presence of human serum has previously been demonstrated [10]. To investigate the effect of serum antibodies only and exclude the effect of other serum components, we use purified human immunoglobulin (IVIg). Serial dilutions of IVIg neutralized RSV (0% normalized infection) at high antibody concentrations and then facilitated up to 300% infection compared to the condition without antibodies (100% normalized infection) in monocytic, FCGR-carrying THP-1 cells (Figure 2A, arrow points to maximum infection enhancement). Commercially available pooled human AB serum and a pool of cord blood plasma, both containing non-immunoglobulin components, showed similar titration curves. Maximum ADE was similar for the three antibody solutions (Figure 2B). To confirm that ADE is mediated by antibody–FCGR interactions in THP-1 cells, FLIPr–like protein was supplemented, which blocks FCGR-mediated Fc–binding. FLIPr–like protein reduced ADE from 340% to 125% infection for IVIg (Figure 2C). To assess whether ADE can occur in primary cells, we performed an ADE assay using human PBMCs. Also in primary immune cells, RSV infection was enhanced up to 230% (Figure 2D).
FIGURE 2  ADE in monocytic cell line THP-1 and primary cells ·

A. RSV neutralization assay using FCGR–carrying THP-1 cells in the presence of serially diluted IVIg, human serum, or cord blood. B. ADE is reported for the dilution at which infection is maximal (depicted by the arrow in A). C. Fc–binding sites of FCGRs were blocked by adding 3 µg/mL staphylococcal FLIPr–like protein to THP-1 cells during RSV neutralization assay. D. RSV neutralization assay using cells that were isolated from human blood (human PBMCs). One representative graph of three PBMC donors is depicted. For other graphs, the means and SD of ≥ 3 individual experiments.
FIGURE 3  Reduction of neutralization of RSV in FCGR2a-transduced Vero cells.

RSV neutralization assays using parental and FCGR2a-transduced Vero cells (Vero2a) in the presence of serially diluted IVIg, human serum or cord blood (A–C). The ΔPRNT50 is a measure for the reduction of neutralization titer in Vero2a cells (D) calculated by dividing the PRNT50[Vero] by PRNT50[Vero2a] (depicted by the arrow in A–C). Means and SD of ≥ 3 individual experiments.
**FIGURE 4** ADE and ΔPRNT50 by maternal antibodies.

ADE (A) and ΔPRNT50 (B) were determined for 15 individual cord blood samples. Statistical dependence between ADE and ΔPRNT50 was tested by Spearman’s correlation (C).
ΔPRNT50 on FCGR2a-expressing Vero cells as measure for FCGR-mediated reduction of neutralization

Eliciting RSV-neutralizing antibodies is the primary goal of RSV vaccination efforts. Intriguingly, the presence of FCGRs in target cells mostly reduces the RSV neutralization capacity of monoclonal antibodies [12]. Preventing Fc receptor-interactions restored RSV neutralization by IVIg in THP-1 cells (Figure 2C). We previously demonstrated that the impact of FCGRs on RSV neutralization can be measured in Vero cells that stably express FCGR2a (Vero2a) [12]. In this study, titration series of IVIg, pooled human AB serum, and pooled cord blood plasma revealed a 4-fold reduced neutralization on Vero2a compared to parental Vero cells (Figure 3A-D). Although reduction in neutralization can be measured, FCGR-expressing Vero cells show no ADE, which is in line with observations for dengue virus [25].

Inter-individual variation in ADE and ΔPRNT50

To further analyze ADE or ΔPRNT50 and any link to disease symptoms, we first assessed whether it would be feasible to distinguish between individuals with different capacities of ADE and ΔPRNT50. We tested the two Fc-mediated effects in cord blood from 15 individual donors. Most samples showed about 400% ADE compared to infection in the absence of antibodies (Figure 4A). The ΔPRNT50 showed a larger individual variation with up to 10-fold reduction of RSV neutralization in Vero2a cells (Figure 4B). Interestingly, the ΔPRNT50 was unrelated to ADE (Figure 4C), indicating that they are independent effects.

ADE and ΔPRNT50 do not correlate with RSV neutralization or anti-RSV IgG levels.

To investigate a possible contribution of classical antibody characteristics to ADE or ΔPRNT50, both phenotypes were compared to two classical serological parameters: RSV neutralization in Vero cells (Figure 5A, B) and amount of RSV–specific IgG (Figure 5C, D). Slightly more reduction of neutralization was detected when RSV neutralization titers were highest (Figure 5B), but this was not significant. No associations between any of the other parameters were detected.
**FIGURE 5**  
ADE and ΔPRNT50 are independent of classical serological parameters. 

ADE and ΔPRNT50 of 15 individual cord blood samples were compared to the RSV neutralization titer in Vero cells (A, B) or the relative amount of RSV-specific IgG (C, D). Associations were tested with Spearman’s correlation.
ADE in primary cells from cotton rat lungs

ADE of RSV infection has not been demonstrated in vivo, but the inoculation of mice with RSV in complex with antibodies has been shown to reshape the immune response compared to RSV alone [26]. Natural RSV infections target pulmonary tissue, therefore we tested cells derived from lungs of naive cotton rats. To determine the type of cells in these lung cell isolates, we stained the cells for epithelial marker pan–cytokeratin [22] and T cell marker CD3ε [23]. Only 3% of cells were of epithelial origin, whereas 35% of cells were CD3ε positive (data not shown). This indicated that a large proportion of isolated cells were immune cells. Pools of plasma from RSV-challenged animals enhanced RSV infection in these cells by 330% (Figure 6A). No ADE was induced when serum from naive cotton rats was used, indicating the requirement of RSV-specific antibodies for ADE.

Characterization of FI–RSV–induced antibodies in immunized cotton rats

As cotton rat antibodies could enhance RSV infection in primary cells, we wanted to understand their effect on RSV disease. It has been shown that antibodies generated as part of active immunity induced in infants by FI–RSV vaccination contribute to enhanced pathology [13]. The cotton rat provides a model for FI–RSV–induced RSV pulmonary pathology [15]. Therefore, we investigated whether reduced neutralization and enhanced RSV infection of FCGR-bearing cells relates to pathology in the FI–RSV cotton rat model.

Animals were immunized with FI–RSV for non–protective antibodies or recombinant live–attenuated RSV (rRSV) for protective immunity. First, RSV–specific antibodies in cotton rats were characterized. RSV neutralization in Vero cells demonstrated low, dose–dependent titers of neutralizing antibodies in all FI–RSV–immunized animals, whereas neutralization capacity of plasma from animals immunized with rRSV was high (Figure 6B). Correspondingly, RSV–specific antibody levels were low after FI–RSV and high after rRSV immunization (Figure 6C).

Next, the ability of cotton rat antibodies to cause ADE in the human THP–1 cell model was assessed. Plasma of all immunized animals caused ADE in THP–1 cells (Figure 6D). Infection was increased by 580% for plasma from rRSV–immunized animals and 380% after FI–RSV immunization. The dilution at which maximum ADE was measured, was low for the low–dose FI–RSV–, intermediate for high–dose FI–RSV– and highest for
FIGURE 6  ADE by RSV–specific cotton rat antibodies.

A. RSV neutralization assay using cells that were isolated from cotton rat lungs. Plasma pools were from naive (−) or wild-type RSV–infected (+) cotton rats. B–D. Four animals per group were immunized with live RSV (rRSV) or medium (mock), or with a low or high dose formalin–inactivated RSV (Fi–RSV) or formalin–treated medium (Fi–mock) and subsequently challenged with RSV. RSV neutralization titer (B) and RSV–specific IgG (C) of cotton rat plasma on day 4 after challenge. (D) RSV neutralization assay using THP-1 cells to determine the maximum ADE of cotton rat plasma.
rRSV-immunized animals. This corresponds to RSV neutralization titers and RSV-specific antibody levels. ΔPRNT50 could not be determined for the cotton rat sera, due to a lack of neutralizing capacity making it impossible to calculate a PRNT50.

**Serum of cotton rats with lung pathology causes ADE in the absence of neutralization**

To assess the relationship between ADE of RSV infection and severity of pulmonary pathology in cotton rats, pathology after RSV challenge of FI–RSV– or rRSV–immunized cotton rats was studied. Affirming previous studies, immunization with rRSV protected the animals and resulted in little pathology after challenge (Figure 7A). Both FI–RSV–immunized groups showed elevated lung pathology compared to the group treated with rRSV or the mock vaccine. Moreover, the level of subepithelial, peri–arterial, and bronchiolar infiltrate was higher in FI–RSV–immunized cotton rats than in the control groups in our study. This is in accordance with the increased lymphocyte infiltration in FI–RSV–vaccinated children and animals in previous studies [27, 28].

Next, the ADE and RSV neutralization titer of cotton rat sera were compared to the sum of pathology scores (cumulative pathology score). Sera from mock–immunized animals were excluded. The capacity to induce ADE was present in all sera that contained RSV–specific antibodies, independent of the pathology score. More ADE appears to be linked to less pathology, although the difference in ADE over the range of pathology was small (Figure 7B). Lower pathology score might concur with efficient RSV neutralization and high anti–RSV antibody levels (Figure 6B, C). The latter was confirmed, as pathologic consequences of RSV challenge were high, when RSV neutralization titers were low (Figure 7C). Thus, antibodies from cotton rats with FI–RSV–induced pulmonary pathology were weak neutralizers and capable of causing ADE in vitro.

**ADE and ΔPRNT50 do not correlate with RSV disease outcome in hospitalized infants**

As ADE measured in vitro might relate to severe disease in vivo in absence of neutralization, a possible association between severity of symptoms in infants and ADE or ΔPRNT50 was investigated. Plasma from hospitalized patients with an acute primary RSV infection was analyzed. As this is not
**FIGURE 7** Lung pathology in cotton rats that show antibody-dependent enhancement (ADE) in vitro in the absence of neutralization.

Four animals per group were immunized with live RSV (rRSV) or medium (mock), or with a low or high dose formalin-inactivated RSV (FI-RSV) or formalin-treated medium (FI-mock) and subsequently challenged with RSV. A. Histopathologic lung pathology scores. B, C. Cumulative pathology scores, calculated by the sum of individual pathology scores, were compared with ADE and PRNT50 by Spearman’s correlation.
FIGURE 8 ADE and ΔPRNT50 by plasma from patients with different severities of RSV-mediated disease symptoms.

RSV neutralization assays using plasma samples from patients with moderate or severe RSV-mediated disease and healthy control patients (uninfected). Maximum ADE (A) and plasma titer at which ADE was maximal (B) were compared to disease severity. (C) ΔPRNT50 was compared to disease severity. Statistical analyses employed one-way ANOVA for comparison between the three disease groups (**p < 0.01).
a prospective study, the ideal control group of RSV-infected children with mild symptoms was not available, as blood of these children is not routinely sampled. A common practice in RSV research is to use age-matched uninfected infants as control. One assumes that this group is not protected and if healthy infants become RSV infected, a vast majority of 98–99% would develop only mild symptoms [29]. Analysis of the plasma samples showed that RSV neutralization and epitope specificity did not correlate with severity of symptoms [6]. The lack of this correlation indicates there is no bias for antibody concentration or neutralization in each disease group. Therefore, this is an ideal sample set to assess ADE and ΔPRNT50 on FCGR-expressing cells.

First, maximum ADE of RSV infection by plasma from uninfected, moderate, and severe groups was examined on THP-1 cells. ADE ranged from 400 to 1000% of infection, showing a greater variation than in cord blood samples. However, disease severity and average ADE were not related (Figure 8A). Next, the plasma titer at which ADE was maximal was calculated for all samples. The infants with moderate RSV disease had a significantly higher ADE titer than the uninfected controls, but no correlation with severe disease could be found (Figure 8B). On Vero2a cells, the ΔPRNT50 ranged from 1- to 4-fold reduced neutralization (Figure 8C), but the average ΔPRNT50 was similar in all groups. We also checked correlation between ADE, ΔPRNT50 and age, but no relation was found (data not shown).

**DISCUSSION**

The serum concentration and neutralization capacity are of primary concern when assessing the antiviral activity of antibodies. However, antibodies have many other functions, mediated by the Fc region, that play a role in the immune response. On FCGR-bearing cells, the antibody–FCGR interaction may lead to antibody–dependent enhancement (ADE) of infection or to reduction of neutralization (ΔPRNT50). We performed this study, because we were intrigued by the fact that most severe RSV cases occur in RSV patients under the age of six months, when relatively high levels of RSV-neutralizing matAbs can be found. Moreover, infants that experienced vaccine-enhanced disease after the 1960’s FI–RSV trials had an RSV–specific antibody response, albeit non–protective. Both these
maternal antibodies and FI-RSV-induced antibodies could therefore have a detrimental role in RSV-mediated disease when not neutralizing enough to protect infants from infection.

Generally, Fc-mediated effector functions of RSV-specific antibodies are not considered when studying acute RSV disease, even though some studies demonstrated ADE of RSV infection *in vitro* including an increase of ADE after RSV infection [9–11, 30]. RSV-specific matAbs may cause ADE and reshape the immune response against RSV [26, 30, 31]. Interestingly, matAbs against dengue virus (DenV) have been suggested to worsen DenV disease in infants [32, 33].

We investigated whether FCGR interactions of RSV-specific serum antibodies relate to the severity of RSV disease symptoms in different settings. ADE of RSV infection and ΔPRNT50 were studied in FCGR-bearing cells using plasma from naive children with severe RSV disease, cord blood plasma, and plasma from FI-RSV-immunized cotton rats showing pathology. There are clear differences between severe RSV disease in naive infants that fully depend on maternal antibodies for RSV-specific immunity and FI-RSV vaccine induced pathology that is mediated by pathogenic vaccine-induced antibodies in the presence of an unbalanced cellular response [34]. For example, FI-RSV vaccine-enhanced pulmonary pathology is associated with inadequate cellular immune responses [35–37]. Another well-established difference is the low RSV neutralization capacity of serum antibodies induced by FI-RSV vaccination [13, 38, 39]. The use of sera from two different types of RSV disease, allowed us to assess the ADE and neutralization-reducing characteristics for both passively acquired matAbs and actively acquired antibodies in a vaccination setting.

ADE was seen for plasma of both healthy and severely ill infants in different degrees, but we did not find a correlation between ADE and RSV disease. There are several considerations to be made. First, it is well described that ADE is related to more disease severity in DenV [40–42]. However, there are fundamental differences between DenV and RSV infection. Only a single serotype exists for RSV, whereas heterotypic, non-neutralizing antibodies exacerbate DenV disease. In addition, the primary target cells for RSV are lung epithelial cells, in contrast to immune cells for DenV. Interestingly, there is a growing body of evidence that RSV also infects immune cells. Recently, infection of several subsets of immune cells has been found in
infants with severe RSV disease [43, 44]. Infection of these FCGR-carrying immune cells may well be aided by ADE, which could affect the immune response and subsequent pathology, as in DENV infections.

Second, our study demonstrates that ADE of RSV in vitro occurs only at non-neutralizing concentrations of antibodies. Even minute amounts of antibodies that fail to neutralize can mediate ADE. Possibly, such conditions existed in some of the infants in our study: bronchial antibody concentrations can be about 100-times lower than in circulation, where we measured them [45–47] and RSV-specific matAbs can decline rapidly with a half-life of approximately one month [3]. Such the age-dependent decline of matAbs has been described in DenV infections and has been suggested to increase the risk for severe disease [48, 49]. Interestingly, in contrast to most RSV-specific monoclonal antibodies, neutralization by palivizumab improves in cells expressing FCGR [12]. This unique feature of the therapeutic monoclonal antibody might explain its clinical efficacy.

Third, our findings on maternal antibodies from children with varying levels of RSV disease were different from findings on antibodies from cotton rats that show vaccine-enhanced pulmonary pathology mediated by FI–RSV-induced immune responses. In children, neither ADE nor reduced neutralization did correlate with disease severity, but in FI–RSV-immunized cotton rats high ADE capacity in vitro coincides with pathology in the absence, but not in the presence of neutralization, as seen for rRSV-immunized animals. This suggests that ADE cannot induce severe pathology in presence of neutralization, but should be considered in non-neutralizing conditions.

Although various reports do emphasize the importance of antibodies in vaccine-enhanced RSV disease [13, 38, 39, 50], it is generally accepted that FI–RSV vaccine enhanced pathology results from an unbalanced immune response and a combination of aberrant humoral and cellular responses. In animal models, especially CD4+ T cells seem to be important mediators of pathology [35, 37]. Interestingly, such a requirement for the FCGR-carrying cellular arm of the immune system might explain why RSV-specific antibodies have a low probability of influencing disease through ADE, in the absence of a cellular response, such as is the case with maternally derived antibodies. It would also be in accordance with data from a previous study, in which it was shown that passive transfer of serum from FI–RSV-immunized cotton rats to naïve animals was not sufficient to
induce vaccine-enhanced disease [51]. This does not exclude that such antibodies, in their original setting, thus acquired through immunization, in combination with immune cells, could play a role in disease, but only when they are non-neutralizing, as is the case in FI–RSV–immunized animals.

Our data show that severe pulmonary pathology in the cotton rat FI–RSV vaccination model occurs in the presence of antibodies that are non-neutralizing and cause ADE in vitro. This relation may also exist in humans: since the initial demonstration that the formalin–inactivated vaccine enhanced disease severity in children, ADE has been considered as an explanation [52]. FI–RSV–immunized infants developed non-neutralizing antibody titers, a prerequisite for ADE, possibly because formalin treatment of RSV modified the viral surface epitopes [11, 53]. Correspondingly, vaccine–enhanced pathology was partially attributed to increased levels of non-neutralizing antibodies and immune complex deposition in previous reports [13, 38, 39, 50]. This could lead to augmented RSV infection of immune cells that carry FCGR and/or reshaping of the immune response by FCGR signaling.

Recently, it has been shown that binding of immune complexes to FCGR is not enough to induce ADE for Ebola virus [54]. Downstream signaling pathways were required to observe enhancement of infection. This may explain why we were unable to detect ADE on the FCGR–transduced Vero cells. Most likely, the FCGRs expressed on Vero cells are non-functional in signaling; they only mediate the binding of immune complexes and cannot internalize the RSV–antibody immune complexes.

We show that there is ADE capacity in vitro when RSV–mediated pathology and RSV–specific antibodies are present. However, additional research is needed to elucidate the involvement of ADE in infection of immune cells in vivo during natural RSV infection or after vaccination. We did not see a correlation between ADE in vitro and disease severity for the infants in our cohort with the assays described in this paper. However, multiple additional antibody functions that may have immunomodulatory effects have not been tested yet (reviewed in [55]). In addition, using THP–1 cells as a model system only demonstrates the effect on monocytes, whereas other immune cells express different combinations of FCGRs and could react differently. It could well be that ADE is part of a set of conditions, including the status of the infant’s immune system or airway size that determine the outcome of RSV infection.
In summary, antibodies can favor ADE and have reduced neutralization capacity in Fc-receptor carrying cells. Antibodies from FI-RSV-immunized cotton rats showed no neutralization and were able to induce ADE in vitro. In infants, ADE and reduced neutralization by matAbs in vitro did not relate to severity of RSV disease symptoms, although all tested human plasma did have the capacity to cause ADE. There may be different effects from passively transferred matAbs compared to antibodies induced by direct vaccination, particularly when they are non-neutralizing as we show for those induced by formalin–inactivated vaccines. This could lead to different outcomes of ADE.

This report shows that ADE should not be ignored as a possible player in RSV infection. Most importantly, the induction of non-neutralizing antibodies or sub-neutralizing antibody titers should be avoided. It remains a challenge to find out whether immune cells are infected through ADE of RSV infection in vivo. It should also be noted that RSV infection of immune cells may have multiple effects, in a range from (hyper)activation to shutting down cellular effector functions. Any of these effects could lead to the known contribution of immune cells to immunopathology in severe RSV-mediated disease. Determining whether ADE of RSV infection is possible in these cells in vivo and how these cells react may provide clues to why severe RSV disease occurs in certain cases and how we can prevent the burden of disease in these young infants.
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PART III

VIRAL INFECTION OF NK CELLS AND THE INFLUENCE ON EFFECTOR FUNCTIONS
RESPIRATORY SYNCYTIAL VIRUS INFECTS PRIMARY NEONATAL AND ADULT NATURAL KILLER CELLS AND AFFECTS THEIR ANTIVIRAL EFFECTOR FUNCTION

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ABSTRACT

Background
Respiratory syncytial virus (RSV) is a major cause of severe acute lower respiratory tract infections in infants. Natural killer (NK) cells are important anti-viral effector cells that likely encounter RSV in the presence of virus-specific (maternal) antibodies. Since NK cells potentially contribute to immunopathology, we investigated whether RSV affects their anti-viral effector functions.

Methods
We assessed the phenotype and functionality of primary neonatal and adult NK cells by flow cytometry after stimulation with RSV or RSV–antibody complexes.

Results
We demonstrate for the first time that RSV infects neonatal and adult NK cells in vitro. Pre-incubation of virus with sub-neutralizing concentrations of RSV–specific antibodies significantly increased the percentage of infected NK cells. Upon infection, NK cells were significantly more prone to produce IFN-γ, while secretion of the cytotoxicity molecule perforin was not enhanced.

Conclusions
Our findings suggest that (antibody-enhanced) RSV infection of NK cells induces a pro-inflammatory rather than a cytotoxic response, which may contribute to immunopathology. Considering that most RSV vaccines currently being developed aim at inducing (maternal) antibodies, these results highlight the importance of understanding the interactions between innate effector cells and virus-specific antibodies.
INTRODUCTION

Human respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract disease in infants [1]. There are currently no market-approved vaccines or antivirals available against this virus. The estimated global burden of RSV-associated severe acute lower respiratory infection was 33.1 million in 2015, with an estimated 118,200 deaths in children below 5 years of age [2]. Hospitalization for severe RSV-mediated disease peaks between six weeks and six months of life [3], when infants mainly depend on maternal antibodies and their innate immune system for protection against infectious diseases. Despite extensive research efforts, the immunological determinants of severe RSV-mediated disease remain elusive.

Natural killer (NK) cells are innate lymphocytes that play an important role in the control of viral lung infections. Within days after infection, large numbers of NK cells are recruited to the lung and become activated [4,5]. NK cells have multiple mechanisms to combat viral replication: 1) death receptor–mediated cytolysis to kill virus–infected target cells, 2) production of pro-inflammatory cytokines with antiviral activity (e.g. IFN-γ), and 3) antibody-dependent cell-mediated cytotoxicity (ADCC), in which NK cells bind antibody-coated virus–infected target cells via CD16/Fc gamma receptor III (FcγRIII) followed by target cell lysis.

The role of NK cells during RSV-induced disease is still unclear. In mice, increased numbers of NK cells are present in the lungs early after RSV infection [4–6]. In this model, the presence of NK cells is sufficient to eliminate RSV infection [7] and depletion of NK cells significantly increases viral loads [8]. However, increasing evidence suggests that NK cells also contribute to inflammatory lung injury, for example via the production of IFN-γ [5,8,9].

There are contradictory reports on NK cells in humans during severe RSV infection. In infants, the proportion of NK cells has been reported both to be decreased [10–13] or increased [14,15] in comparison with healthy controls or infants with mild symptoms. NK cell gene expression in whole blood was reported to be downregulated in infants with severe RSV disease compared to controls [16]. Therefore, definitive conclusions about the role of NK cells in RSV infection and disease cannot be drawn from the data currently available.
In this study, we investigated whether interaction of RSV or RSV-antibody complexes with NK cells affects their function. Interestingly, we found that RSV infects NK cells and that infection influenced the effector function of both neonatal and adult NK cells. RSV-infected NK cells were more prone to produce IFN-γ than uninfected cells, while the percentage of perforin-secreting cells was not increased. We show that pre-incubation of RSV with sub-neutralizing concentrations of virus-specific antibodies increases the number of infected and, hence, IFN-γ secreting NK cells. We propose that (antibody-enhanced) infection of NK cells with RSV may contribute to immunopathology, through induction of a pro-inflammatory rather than a cytotoxic response in these cells.

**MATERIALS AND METHODS**

**Cells and viruses**

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers at the National Institute for Public Health and the Environment (RIVM, the Netherlands). Cord blood mononuclear cells (CBMC) from umbilical cords of healthy neonates born by caesarian section were collected at Radboudumc Nijmegen (the Netherlands). Blood was collected in heparin tubes and the mononuclear fraction was isolated by density gradient centrifugation (Lymphoprep, Nycomed). NK cells were purified by negative selection using a CD56+ NK cell isolation kit (Miltenyi Biotec). In all experiments, NK cells were gated as the CD3(-), CD56(+) population. Isolated cells were cultured in IMDM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (hiFCS), 1% penicillin/streptomycin/glutamin (PSG, Gibco), and 5 ng/mL recombinant IL-15 (Biolegend). CBMCs were stored at −135°C and thawed before NK cell isolation.

Vero cells (ATCC-CCL81) were propagated in DMEM supplemented with 5% hiFCS and 1% PSG. Human chronic myelogenous leukemic K562 cells (kind gift from Jeannette Cany, Radboudumc Nijmegen, the Netherlands) were propagated in IMDM, supplemented with 10% hiFCS and 1% PSG.

Recombinant RSV-X and RSV-X-GFP7 (encoding green fluorescent protein) were propagated in Vero cells as described before [17,18]. Virus stocks were purified between layers of 10% and 50% sucrose by ultracentrifugation. 50% tissue culture infectious dose (TCID50)/mL was determined on Vero cells using the Spearman and Karber’s method [19].
Plaque reduction neutralization test (PRNT)
Using RSV-X-GFP7, a Vero-based PRNT was performed for intravenous immunoglobulin (IVIg, KIOVIG, Baxalta), Palivizumab (SYNAGIS, Abbvie), and the WHO International standard for antiserum to RSV (NIBSC), as described previously [18]. Based on the neutralization curves, the amount of international units (IU) of anti-RSV neutralizing antibodies per mL was calculated for IVIg and Palivizumab using the WHO standard as reference serum (containing 2000 IU/mL).

RSV infection
NK cells were infected with RSV-X-GFP7 or RSV-X by spinoculation for 1 h at 700 x g at 20°C, followed by incubation for 1 h at 37°C. A multiplicity of infection (MOI) of 1 based on titration on Vero cells was used. Next, cells were washed with PBS and replenished with culture medium. For antibody-dependent enhancement (ADE) assays, RSV was pre-incubated with the indicated concentrations of IVIg or Palivizumab for 10 minutes at 37°C, before spinoculation of NK cells. Incubation at 37°C was followed by flow cytometric analysis at the indicated time points using an LSR Fortessa X20 (BD Biosciences). RSV infection was blocked by co-incubation with 100 nM fusion inhibitor (TMC-353121, MCE) [20]. CD16/FcγRIII was blocked by pre-incubation of NK cells with 50 µg/mL anti-CD16 Fab fragments (3G8, Ancell). Infection was measured by GFP expression for RSV-X-GFP7 or with a FITC-conjugated RSV-G antibody (131-2G, Millipore). Productivity of NK cell infection was assessed by TCID50 of the cleared supernatants on Vero cells as described above.

Flow cytometric phenotypic characterization
The following fluorochrome-conjugated monoclonal antibodies were used to phenotypically characterize (RSV-infected) NK cells: CD3–APCA750 (UCHT1, Beckman Coulter), CD16–PacificOrange (3G8, Thermofisher), CD56–ECD (N901, Beckman Coulter), CD85j–PerCP–Cy5.5 (ILT2, LilRB1; GHI/75, BioLegend), CD161–APC (191B8, Miltenyi), CD158a–AF700 (KIR2DL1; 143211, R&D systems), CD158a/h–PC5.5 (KIR2DL1/S1; EB6B, Beckman Coulter), CD158b1/b2,j–PC7 (KIR2DL2/L3/S2; GL183, Beckman Coulter), CD158e1–BV421 (KIR3DL1; DX9, BioLegend), CD159a–APC (NKG2A; Z199, Beckman Coulter), CD159c–PE (NKG2C; 134591, R&D systems), CD244–AF700 (2B4; C1.7, BioLegend), CD314–APC (NKG2D;
ON72, Beckman Coulter), CD335–PC7 (NKp46; BAB281, Beckman Coulter), CD336–PE (NKp44; Z231, Beckman Coulter), CD337–PerCP–Cy5.5 (NKp30; P30-15, BioLegend), RSV–G–FITC (131-2G, Millipore). Cells were measured using a Navios flow cytometer (Beckman Coulter).

**NK activation assay**

At 20 hours post infection (hpi) with RSV or RSV–antibody complexes, the NK cells were incubated for 4 h in the absence or presence of K562 target cells together with Brefeldin A (BD Bioscience) and CD107a–PE/Cy7 antibody (H4A3, Biolegend). Subsequently, cells were stained using the following antibodies: CD56–PE (HCD56, Biolegend), CD3–PerCP (SK7, BD Biosciences), RSV–G–FITC (131–2G, Millipore), IFN–γ–APC antibody (B27, BD Bioscience), perforin–BV421 (B–D48, Biolegend), and fixable viability dye eFluor780 (eBioscience).

**Statistical analysis**

Comparison of two groups or data points was performed by using a non-parametric Wilcoxon signed rank test. Multiple comparisons were analyzed by using a non-parametric Friedman test, followed by Dunn’s multiple comparison test. P values <0.05 were considered statistically significant. All statistical analyses were performed with Prism 7 software (GraphPad).

**Ethics statement**

All blood donors (PBMC) and mothers (CBMC) provided written informed consent.
RESULTS

RSV infects and replicates in primary adult NK cells
To assess the interaction of RSV with NK cells, primary adult NK cells (>95% CD3(−) cells) were spinoculated with RSV-X-GFP7 at a Vero-based MOI of 1. We observed steadily increasing expression of virus-encoded GFP, which is indicative of viral replication. In a time-course experiment, the maximum percentage of GFP-positive NK cells (CD3(−), CD56(+)) was observed at 24 hpi (Figure 1A). The level of RSV infection showed considerable donor variability, and reached a maximum of up to 20% infected NK cells in some donors. The amount of intracellular GFP increased over time as shown by the MFI (Figure 1B). TCID50 assays of the NK cell supernatant showed a decrease in viral titer over time, suggesting that little or no infectious viral particles were released (Figure 1C). Inoculation of NK cells with RSV-X-GFP7 in the presence of a fusion inhibitor (TMC) showed efficient inhibition of NK cell infection (Figure 1D), indicating that viral entry was required for GFP detection and depended on the fusion (F) protein. The TMC vehicle control (DMSO) showed no effect on infection (Supplementary Figure 1A). Moreover, increasing the titer of the inoculum resulted in considerably higher infection rates (Supplementary Figure 1B), suggesting that RSV does not exclusively infect a minor NK cell subpopulation. Altogether, these data show that RSV infects primary adult NK cells in vitro.

Non-neutralizing RSV-specific antibodies enhance NK cell infection
The vast majority of individuals infected with RSV possess RSV-specific antibodies, which vary in concentration and are either maternally-derived through transplacental transfer or induced by previous exposure. NK cells that are recruited to the lung most likely encounter viral particles in complex with these antibodies. Since IgG levels in the lung are lower than those found in serum [21], even neutralizing concentrations of serum antibodies may be accompanied by non-neutralizing antibody levels in the lung. Here we show that, while high antibody concentrations result in virus neutralization, incubation of NK cells with RSV-antibody complexes formed at sub-neutralizing concentrations results in antibody-dependent enhancement (ADE) of infection (Figure 2A).
FIGURE 1  RSV infects primary NK cells in vitro ·
A–B. Flow cytometric analysis showing the percentage (A) or Median Fluorescence Intensity (MFI, B) of GFP-positive cells for adult primary NK cells after spinoculation with RSV-X-GFP7 (MOI = 1). Graphs depict geometric mean and SD of n = 4 donors pooled from two independent experiments.  
C. TCID50 of cleared RSV-infected NK cell supernatant on Vero cells. Graph depicts geometric mean and SD of n = 3 donors from one experiment. The dashed line depicts the retitration of the initial RSV inoculum.  
D. NK cells were spinoculated with RSV-X-GFP7 in the absence or presence of a fusion inhibitor (TMC) and infection was measured by flow cytometry at 20 hpi. Data was pooled from six independent experiments and each set of paired data points represents an individual donor (n = 14). Wilcoxon signed rank test was used for comparison between conditions (***p < 0.001).
FIGURE 2 Antibody-dependent enhancement of RSV infection in NK cells

A. Infection of adult primary NK cells in the presence of RSV-X-GFP7-antibody complexes (with the indicated concentration of IVIg or Palivizumab). Graph depicts the geometric mean and SD of n = 3 donors from one experiment. Dashed line depicts the geometric mean percentage GFP-positive cells in absence of antibodies (no Abs).

B. Infection of NK cells with RSV-X-GFP7 or (sub-) neutralizing RSV-antibody complexes (5 µg/mL or 500 µg/mL IVIg). Data was pooled from six independent experiments and each set of paired data points represents an individual donor (n = 14).

C. Infection of NK cells with sub-neutralizing RSV-antibody complexes (5 µg/mL IVIg) in the absence or presence of a fusion inhibitor (TMC). Data was pooled from six independent experiments and each set of paired data points represents an individual donor (n = 14).

D. Infection of NK cells in the...
absence or presence of sub-neutralizing RSV-antibody complexes (5 µg/mL IVIg) with 50 µg/mL CD16-blocking Fab fragments. Graph depicts the geometric mean and SD of n = 3 donors from one experiment. All GFP measurements were performed by flow cytometry at 20 hpi. Non-parametric Friedman test with Dunn’s multiple comparison test was used for comparisons between multiple conditions (*p < 0.05, ****p < 0.0001). Wilcoxon signed rank test was used for comparison between two conditions (**p < 0.001).

This was shown for both IVIg, naturally containing RSV-specific antibodies, and Palivizumab, an RSV-specific monoclonal antibody targeting the F protein. IVIg showed maximum enhancement around 1 µg/mL, corresponding to 0.0027 IU/mL of anti-RSV neutralizing antibodies. For Palivizumab, maximum enhancement was observed at 0.03 µg/mL, corresponding to 0.0013 IU/mL. The standardized concentration neutralizing antibodies of IVIg and Palivizumab were calculated using the WHO international standard for antiserum to RSV as a reference (Supplementary Figure 1C).

Incubation of NK cells with RSV-antibody complexes formed at sub-neutralizing antibody concentrations (5 µg/mL or 0.014 IU/mL IVIg), resulted in up to 4-fold increased infection compared to the absence of antibodies (Figure 2B). Neutralizing antibody concentrations (500 µg/mL or 1.4 IU/mL IVIg) completely inhibited infection. ADE of infection was completely blocked in the presence of TMC, indicating that viral entry still depended on the F protein (Figure 2C). ADE of NK cell infection seems to involve CD16/FcγRIII, as incubation with CD16-blocking Fab fragments decreased infection in the presence but not in the absence of RSV-specific antibodies, although this difference was not statistically significant (Figure 2D). Spinoculation was used to enhance infection in all experiments, but this had no effect on the fold increase of antibody-enhanced infection compared to regular infection (Supplementary Figure 1D).

Phenotypic characterization of RSV-infected NK cells

Next, we set out to determine the phenotypic characteristics of RSV-infected NK cells using an extensive NK cell receptor panel. Those receptors showing the most pronounced effect upon infection are shown in Figure 3; the remaining receptors are depicted in Supplementary Figure 2. In the presence of sub-neutralizing RSV-antibody complexes, the activation markers NKG2D and NKp44 are downregulated (Figure 3A, B).
RSV INFECTS NK CELLS

**FIGURE 3** Phenotypic characterization of RSV-infected NK cells

A–D. Adult primary NK cells were inoculated with RSV-X or sub-neutralizing RSV-X-antibody complexes (5 µg/mL IVIg) and stained with three different antibody panels for flow cytometric analysis at 20 hpi. RSV-infected and uninfected populations within one well are indicated with (+) and (−), respectively. Geometric mean of fluorescence intensity (MFI) is depicted for the markers with the most pronounced differences compared to mock-infected NK cells: activating receptors (A) NKG2D and (B) NKp44, and KIRs (C) KIR3DL1, which is inhibitory, and (D) KIR2DL2/L3/S2, which can be either activating or inhibiting. Graphs depict the geometric mean and SD of n = 6 donors pooled from two independent experiments. Non-parametric Friedman test with Dunn’s multiple comparison test was used for comparison between multiple conditions (*p < 0.05, **p < 0.01, ***p < 0.001).
The presence of RSV alone has a similar, but non-significant effect. In contrast, the HLA-C-specific killer cell immunoglobulin-like receptors KIR3DL1, which is inhibitory, and KIR2DL2/L3/S2, which can be activating or inhibiting, were more abundantly expressed on RSV-infected NK cells compared to mock-infected cells (Figure 3C, D). Taken together, although the effect on individual markers is not very dramatic, RSV-infected NK cells appear to be skewed towards an inhibitory phenotype.

**Neonatal NK cells are susceptible to (antibody-enhanced) RSV infection**

RSV is known to cause the most severe symptoms in the first months of life [3]. Therefore, the cells that are present at the moment of severe RSV disease in infants may more closely resemble umbilical cord blood NK cells than adult cells. We found that also neonatal NK cells (>88% CD3(-) cells) could be infected by RSV in vitro, and that infection was blocked by the fusion inhibitor TMC (Figure 4A). Neonatal NK cells showed a 6-fold increase in infection upon incubation with RSV–antibody complexes formed at sub-neutralizing antibody concentrations (5 µg/mL IVIg) compared to the no–antibody control (Figure 4B). Neutralizing antibody concentrations (500 µg/mL IVIg) completely inhibited infection. Overall, these results show that neonatal NK cells are comparable to adult NK cells in their susceptibility to RSV infection.

**RSV infection of NK cells induces IFN-γ production**

Since RSV infection of immune cells can have a profound effect on their functionality [22–24], we set out to explore the effect of RSV infection on NK cell functionality. First, we assessed IFN-γ production in four different NK cell populations: mock–infected control cells (1), RSV–exposed infected (2) and uninfected (3) cells, and uninfected cells exposed to RSV in the presence of TMC (4). The gating strategy for the NK cell activation assays is depicted in Supplementary Figure 3. RSV-infected neonatal NK cells showed significantly more IFN-γ–expressing cells compared to all control conditions (Figure 5A). CD107a, a marker of NK cell activity, was also upregulated in RSV–infected NK cells (Supplementary Figure 4). In agreement with the results obtained for infection by RSV in the absence of antibodies, infection of neonatal NK cells by RSV–antibody complexes resulted in even more IFN-γ–expressing cells (Figure 5B). Similar results
FIGURE 4 Neonatal NK cells are susceptible to RSV infection.

Human neonatal NK cells were spinoculated with RSV-X-GFP7 (MOI = 1). A. Percentage infected cells in absence or presence of a fusion inhibitor (TMC) as measured by flow cytometry. Data was pooled from two independent experiments and each set of paired data points represents an individual donor (n = 8). B. Percentage infected cells after inoculation with RSV or RSV-antibody complexes (5 µg/mL or 500 µg/mL IVlg). Data was pooled from two independent experiments and each set of paired data points represents an individual donor (n = 6). Wilcoxon signed rank test was used for comparison between two conditions (***p < 0.001). Non-parametric Friedman test with Dunn’s multiple comparison test was used for comparison between multiple conditions (**p < 0.01).
FIGURE 5  RSV infection of NK cells induces IFN–γ production.

Neonatal (A–B) or adult (C–D) NK cells were infected with RSV–X, inoculated with RSV–X and TMC, or mock–infected. RSV–infected and uninfected populations are indicated with (+) and (–), respectively. At 20 hpi, NK cells were incubated for 4 h with Brefeldin A and subsequently stained for intracellular IFN–γ. A&C. Percentage NK cells positive for intracellular IFN–γ depicted for control NK cells, RSV(+) and RSV(−) cells within one RSV–inoculated well and for NK cells inoculated with RSV and TMC. B&D. Same as (A/C) except for pre–incubation of the viral inoculum with 5 µg/mL IVIg, resulting in ADE. All graphs depict geometric mean and SD of n = 6 (neonatal) or n = 8 (adult) donors pooled from two (neonatal) or four (adult) independent experiments. Non–parametric Friedman test with Dunn’s multiple comparison test was used for comparison between conditions (*P<0.05, **P<0.01, ***P<0.001).
were obtained using adult NK cells (Figure 5C-D). In summary, both neonatal and adult NK cells are prone to produce IFN-γ upon RSV infection, and ADE of infection increases the total number of IFN-γ-positive cells by increasing the number of infected cells.

**RSV infection of NK cells does not enhance perforin secretion**

In addition to IFN-γ secretion, an important NK cell function is the secretion of granzymes and perforins to induce target cell death. Upon encountering a cytotoxicity trigger, e.g. virus-infected or tumor cells, NK cells rapidly release pre-existing granules containing both granzymes and perforins. Secretion of these granules results in a loss of intracellular perforin staining, which can be detected by flow cytometry [25]. This experimental approach allows for the discrimination between infected and uninfected cell responses in the same well, which would not be possible by measuring perforin released in the supernatant. We determined the percentage of perforin-negative cells as a measure for perforin secretion in five different NK cell populations: mock-infected control cells without K562 target cells (1), mock-infected control cells with target cells (2), RSV-exposed infected (3) and uninfected (4) cells with target cells, and uninfected cells exposed to RSV in the presence of TMC with target cells (5).

Perforin secretion was slightly increased in all conditions upon the addition of target cells to neonatal NK cells (Figure 6A). Although RSV-infected NK cells are considerably more prone to produce IFN-γ than control cells (Figure 5A), this was not the case for perforin secretion. When neonatal NK cells were incubated with sub-neutralizing antibody concentrations (5 µg/mL IVIg), only the RSV-negative cells were significantly more likely to secrete perforin than unstimulated cells, possibly due to activation by neighboring opsonized RSV-infected NK cells (Figure 6B).

Since neonatal NK cells are described to be intrinsically less cytotoxic than adult NK cells [26,27], we investigated whether the latter were more reactive in our cytotoxicity assay. Unlike neonatal cells, adult NK cells showed a significant increase in perforin secretion upon addition of target cells, supporting the reportedly low cytotoxic capability of neonatal NK cells (Figure 6C). Strikingly, only RSV-infected NK cells did not show significantly increased perforin secretion upon addition of target cells. ADE of NK cell infection enhanced this effect, resulting in a significant difference in perforin secretion between infected and uninfected NK cells in the same
FIGURE 6  RSV infection of NK cells does not enhance cytotoxicity.

Neonatal (A-B) or adult (C-D) NK cells were infected with RSV-X, inoculated with RSV-X and TMC, or mock-infected. RSV-infected and uninfected populations are indicated with (+) and (−), respectively. At 20 hpi, NK cells were incubated for 4 h with Brefeldin A in the absence or presence of K562 target cells and subsequently stained for intracellular perforin.

A&C. Percentage NK cells negative for intracellular perforin staining depicted for control NK cells (with or without target cells), RSV(+) and RSV(−) cells within one RSV-inoculated well and for NK cells inoculated with RSV and TMC. B&D. Same as (A&C) except for pre-incubation of the viral inoculum with 5 µg/mL IVIg, resulting in ADE. All graphs depict geometric mean and SD of n = 6 (neonatal) or n = 8 (adult) donors pooled from two (neonatal) or four (adult) independent experiments. Non-parametric Friedman test with Dunn’s multiple comparison test was used for comparison between conditions (*P<0.05, **P<0.01, ***P<0.01).
well (Figure 6D). These data suggest that, unlike what was seen for IFN-γ, the secretion of perforin is not enhanced and possibly even reduced in RSV-infected adult NK cells.

**DISCUSSION**

In the past, it was thought that epithelial cells were the only target of RSV [11,28]. However, there is increasing evidence that RSV is also able to infect immune cells in humans [22,23] and viral RNA has been detected in peripheral blood cells during acute infection [29]. In our experiments, infected NK cells did not release infectious viral particles, which is consistent with studies of RSV infection in other immune cells [24,30,31]. Infection of NK cells has been documented before for several unrelated viruses (including HIV, vaccinia, human herpesvirus 6, and influenza) and only some of these establish productive infections [32–35].

Considering that NK cells, recruited to the lungs during infection in infants, likely encounter RSV bound to (sub-neutralizing levels of) virus-specific antibodies, it is probable that Fc gamma receptor (FcγR)-mediated antibody effector functions are activated. Recently, researchers found that antibodies with enhanced binding to CD16/FcγRIII determined disease severity in dengue infection [36]. Moreover, *in vitro* ADE of RSV infection has been shown before by several groups including our own [37–39]. In the current study, we show that in the presence of sub-neutralizing antibody concentrations, infection of NK cells can be enhanced up to 4-fold for adult NK cells and up to 6-fold for neonatal NK cells, resulting in a substantial proportion of infected NK cells *in vitro*. Further research is needed to determine the exact contribution of CD16/FcγRIII to ADE of RSV infection in NK cells.

We show that RSV infection of NK cells results in increased numbers of IFN-γ-producing cells. In mouse models of RSV infection, NK cells were shown to be the most important source of IFN-γ early after infection [4,5]. However, the role of IFN-γ in RSV disease has been much debated. On the one hand, it has been suggested that IFN-γ is involved in acute lung injury, allergic airway disease, and airway obstruction in animal models [5,40,41] and appears to be associated with virus-induced wheezing in humans [42]. On the other hand, decreased IFN-γ levels were found in the blood and in the nasopharyngeal aspirates of mechanically ventilated
infants compared to non-ventilated infants [43,44]. However, it is debatable whether measurements in the blood accurately reflect IFN-γ levels in the lungs during RSV infection. Also, in a more recent study, IFN-γ levels were found to be elevated during RSV bronchiolitis when measured by nasosorption, but not in nasopharyngeal aspirates, showing a clear effect of sampling technique [45]. Considering these findings, it seems that the detrimental effects of IFN-γ are an exacerbated outcome of an intrinsically beneficial role for this molecule in protection against RSV disease. We have shown that RSV infection of NK cells leads to more IFN-γ-producing cells, a potential way through which the virus disturbs the balanced NK cell response. In contrast to the increase in IFN-γ production in RSV-infected NK cells, we did not observe more perforin secretion upon RSV infection. Adult NK cells infected in the presence of RSV-antibody complexes even showed significantly lower perforin secretion than uninfected cells in the same well. This effect is likely only seen in adult NK cells because of the intrinsically lower capacity of neonatal NK cells to elicit a cytotoxic response. This is also evidenced by the almost 10-fold lower MFI for perforin in neonatal compared to adult NK cells (data not shown). Overall, we only observed minor differences between neonatal and adult NK cells. This was an unexpected finding, as earlier studies show more RSV infection in neonatal compared to adult monocytes [46] and severely decreased cytotoxic responses by neonatal NK cells [26,27]. Our experiments were performed with the addition of small amounts of IL-15 (5 ng/mL). This has been shown to increase the functionality of neonatal NK cells [27] and possibly explains the limited differences between adult and neonatal NK cells in our study. Moreover, high variability has been observed between cord blood donors in earlier studies [47] with some donors exhibiting similar or even higher cytotoxicity levels than adult donors.

Of note, in our experiments, CD107a expression correlated with IFN-γ production but not with perforin secretion. This suggests that CD107a is not merely a secretion marker of perforin, but acts as a more general marker for NK cell activity.

Increased IFN-γ production together with a possible inhibition of perforin secretion suggests a shift towards a pro-inflammatory rather than a cytotoxic NK cell state. In addition, the phenotypic characteristics of RSV-infected NK cells suggest skewing towards a less cytotoxic and more inhibitory phenotype. This may impair the cytolytic function of these cells,
as has been shown for other viruses [35,48]. Based on previous reports and the data described in this paper, we propose that the pro-inflammatory response of RSV-infected NK cells may contribute to the development of RSV-mediated severe disease. Notably, the formalin-inactivated RSV vaccine that caused enhanced disease upon natural infection, induced primarily non-neutralizing antibodies [49]. Our data support the idea that these antibodies may have contributed to enhanced inflammation [50].

For future research, it would be interesting to investigate whether NK cell functionality differs between infants with severe RSV disease and healthy infants. Obtaining NK cells from the lung is however problematic due to ethical considerations and the limited time window for sampling. In addition, peripheral blood of RSV-infected hospitalized infants only contains low numbers of NK cells [10,11,13], which makes it difficult to study these cells. It would be very interesting to compare neonatal umbilical cord blood NK cells from infants who later develop severe RSV-mediated disease with cells from infants that did not suffer severe disease upon infection.

To conclude, we have shown that RSV-infected NK cells are more prone to produce IFN-γ than uninfected cells, whereas the cytotoxic response is not increased. This combination may contribute to RSV immunopathology in vivo, but more research is needed to support this hypothesis. Moreover, we show that Fc-mediated effector functions like ADE of infection can have a profound effect on the immune response and a potential role in the development of disease. Our findings contribute to the understanding of the potential mechanisms responsible for the development of severe RSV disease and highlight the critical need to further characterize the role of NK cells and RSV-antibody complexes therein, as this will assist in the development of safe and effective RSV vaccines.

Supplementary Materials are available online at:
https://academic.oup.com/jid/article/219/5/723/5106664#supplementary-data
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VIRAL INFECTION OF HUMAN NATURAL KILLER CELLS

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ABSTRACT

Natural killer (NK) cells are essential in the early immune response against viral infections, in particular through clearance of virus-infected cells. In return, viruses have evolved multiple mechanisms to evade NK cell-mediated viral clearance. Several unrelated viruses, including influenza virus, respiratory syncytial virus, and human immunodeficiency virus, can directly interfere with NK cell functioning through infection of these cells. Viral infection can lead to immune suppression, either by downregulation of the cytotoxic function or by triggering apoptosis, leading to depletion of NK cells. In contrast, some viruses induce proliferation or changes in the morphology of NK cells. In this review article, we provide a comprehensive overview of the viruses that have been reported to infect NK cells, we discuss their mechanisms of entry, and describe the interference with NK cell effector function and phenotype. Finally, we discuss the contribution of virus-infected NK cells to viral load. The development of specific therapeutics, such as viral entry inhibitors, could benefit from an enhanced understanding of viral infection of NK cells, opening up possibilities for the prevention of NK cell infection.
INTRODUCTION

Natural killer (NK) cells are innate lymphocytes that represent the first line of defense against tumor cells and viral infections [1,2]. The importance of NK cells in the antiviral immune response is underscored by the increased susceptibility to viral diseases of patients with a congenital NK cell deficiency. Although NK cell deficiencies are rare, multiple cases have been described in which increased susceptibility to numerous herpesviruses is shown, which has been extensively reviewed elsewhere [3].

NK cells have multiple mechanisms to kill virus-infected cells, including the engagement of extracellular death receptors and exocytosis of cytolytic granules [4]. To mediate cytolysis through engagement of death receptors expressed on target cells, NK cells express multiple extracellular ligands, including Fas ligand (FasL) and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [5]. Viral infection, for example by cytomegalovirus (CMV) and encephalomyocarditis virus (EMCV) [4], can induce the expression of death receptors on infected cells, which can subsequently interact with FasL and TRAIL on NK cells, resulting in apoptosis of the target cell. The other route to induce cytotoxicity is through the release of stored cytolytic granules that contain perforin and granzymes that enter the target cell and trigger apoptosis through caspase-mediated signaling pathways [4]. In addition to cytotoxicity, NK cells contribute to the antiviral response through the release of a wide range of proinflammatory cytokines with antiviral activity [6].

Activation of NK cells is regulated by a balance in the engagement of its activating and inhibitory receptors in combination with the presence of certain cytokines. Together, these stimuli determine the type and strength of NK cell activity [7].

Healthy cells inhibit NK cell activation mainly through the expression of major histocompatibility complex class I (MHC–I) molecules, which interact with inhibitory receptors present on the NK cell surface. Inhibitory NK cell receptors that ligate to MHC–I include killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs) [7]. This inhibitory receptor-mediated signaling is essential to counteract activating signaling in order to protect against NK cell over-activity. Some viruses are known to downregulate surface expression of MHC–I to interfere with the presentation of viral antigens, thereby escaping detection by the
adaptive immune system [8]. Although this immune evasion strategy is effective in preventing recognition by T cells, decreased MHC-I expression promotes the recognition and clearance of virus-infected target cells by NK cells [9]. The concept of target cell recognition via the absence of inhibitory MHC-I engagement is known as the “missing-self” hypothesis.

The activating receptors that are expressed by NK cells facilitate activation upon detection of viral or stress-induced ligands on target cells. For example, the natural cytotoxicity receptors (NCRs, including NKp46, NKp44, and NKp30 are known to bind viral glycoproteins [10,11], allowing for activation of NK cells upon detection of infected cells. In addition, NK cells are activated through binding to antibody-opsonized target cells with Fc-γ receptor IIIA (FcγRIIIA), which induces antibody-dependent cell-mediated cytotoxicity (ADCC).

Due to the important role of NK cells in the early antiviral immune response, viruses have evolved numerous strategies to evade NK cell effector functions. One of these evasion strategies is the manipulation of NK cells through direct infection. In this review, we provide a comprehensive overview of the viruses that have been reported to infect NK cells. We discuss their mechanisms of entry, describe how they affect NK cell function, and indicate which viruses deplete NK cells through the induction of apoptosis. Moreover, we address the contribution of infected NK cells to viral load.

**ENTRY MECHANISMS**

Viruses have evolved many mechanisms to enter host cells. The best-known mechanism is entry through binding to specific receptors, which either leads to fusion directly at the plasma membrane, or to entry following clathrin- or caveolin-dependent endocytosis of the viral particle. Additionally, viruses may require direct cell–cell interactions for infection. Finally, viral entry may occur through macropinocytosis, which is the nonspecific uptake of extracellular material. Hence, non-specific binding of virus can lead to internalization, even in the absence of specific viral entry receptors on the target cell. Macropinocytosis has been described for multiple viruses [12], but to date this has not been identified as a strategy to enter NK cells. The different entry mechanisms that have been shown for viral infection of NK cells are described below and summarized in Table 1.
## TABLE 1 Overview of viruses known to infect natural killer (NK) cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Entry Mechanism</th>
<th>Modulation of NK cells</th>
<th>Productive infection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torque teno virus (TTV)</td>
<td>Anelloviridae</td>
<td>U</td>
<td></td>
<td>Y</td>
<td>[13]</td>
</tr>
<tr>
<td>Human pegivirus (HPgV)</td>
<td>Flaviviridae</td>
<td>U</td>
<td>↑ Survival ↓ IFN-γ production</td>
<td>U</td>
<td>[14]</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV)</td>
<td>Herpesviridae</td>
<td>U</td>
<td>N</td>
<td></td>
<td>[15]</td>
</tr>
<tr>
<td>Epstein Barr virus (EBV)</td>
<td>Herpesviridae</td>
<td>Acquisition of receptor after cell–cell interaction</td>
<td>Morphological changes Transformation NK cell malignancies</td>
<td>N [16–21]</td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus (HSV)</td>
<td>Herpesviridae</td>
<td>Cell–cell interaction with HSV-infected fibroblasts</td>
<td>U</td>
<td>U</td>
<td>[22]</td>
</tr>
<tr>
<td>Human herpes–virus 6 (HHV–6)</td>
<td>Herpesviridae</td>
<td>↑ CD4 expression</td>
<td>U</td>
<td></td>
<td>[23]</td>
</tr>
<tr>
<td>Varicella zoster virus (VZV)</td>
<td>Herpesviridae</td>
<td>Cell–cell interaction with VZV-infected epithelial cells</td>
<td>↑ CD57 expression ↑ Chemokine receptors ↓ CD56 expression ↓ FcyRIII expression</td>
<td>Y [24]</td>
<td></td>
</tr>
<tr>
<td>Influenza A virus (IAV)</td>
<td>Orthomyxoviridae</td>
<td>Clathrin– and caveolin–dependent endocytosis</td>
<td>↑ Apoptosis ↓ Cytotoxicity ↓ Cytotoxicity receptors ↓ Cytokines and chemokines</td>
<td>N [25–29]</td>
<td></td>
</tr>
<tr>
<td>Measles virus (MV)</td>
<td>Paramyxoviridae</td>
<td>U</td>
<td>↓ Cytotoxicity</td>
<td>U</td>
<td>[30]</td>
</tr>
<tr>
<td>Respiratory syncytial virus (RSV)</td>
<td>Pneumoviridae</td>
<td>Possibly macropinocytosis</td>
<td>↑ IFN–γ production ↑ KIR expression ↓ Cytotoxicity ↓ Cytotoxicity receptors</td>
<td>N [31,32]</td>
<td></td>
</tr>
<tr>
<td>Vaccinia virus (VV)</td>
<td>Poxviridae</td>
<td>Cell–cell interaction</td>
<td>↑ KIR signaling ↓ Cytotoxicity</td>
<td>N [33,34]</td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis virus (VSV)</td>
<td>Rhabdoviridae</td>
<td>U</td>
<td></td>
<td>Y</td>
<td>[35]</td>
</tr>
<tr>
<td>Human immuno–deficiency virus 1 (HIV–1)</td>
<td>Retroviridae</td>
<td>Receptor–mediated entry</td>
<td>↑ Apoptosis</td>
<td>Y [36–40]</td>
<td></td>
</tr>
<tr>
<td>Human T–lymphotropic virus (HTLV)</td>
<td>Retroviridae</td>
<td>Cell–cell interaction with T cells</td>
<td>↑ Proliferation ↑ Survival</td>
<td>U [41–43]</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** FcγR, Fc gamma receptor; IFN, interferon; KIR, killer cell immunoglobulin-like receptor; MHC, major histocompatibility complex; Y = yes N = No U = Unknown
Receptor-mediated viral entry

One of the entry pathways for viruses is through binding of cellular receptors by viral proteins, which leads to receptor-mediated entry. NK cells express multiple receptors that are known to be used for virus entry. Additionally, it has been described that NK cells acquire entry receptors from infected cells, either through direct contact via the immunological synapse or by exosome transfer (extensively reviewed in [44]).

Human influenza A virus (IAV) is an enveloped virus that has been shown to directly infect both human and mouse NK cells in vitro [25,26]. In vivo infection of NK cells was demonstrated in several mouse studies [26,45]. Viral entry by IAV into NK cells from human peripheral blood mononuclear cells (PBMCs) was found to be mediated by clathrin- and caveolin-dependent endocytosis [25]. IAV virions generally infect respiratory tract epithelial cells by binding of viral hemagglutinin (HA) protein to α-2,3 and/or α-2,6 linked terminal sialic acids, which are found in abundance on the surface of respiratory tract epithelial cells [46]. It has been confirmed that both forms of sialic acids are also present on the surface of NK cells [26]. In addition, the α-2,6 terminal sialic acids are present on activating NK cell receptor NKp46 [11]. Indeed, free HA binding and internalization into NK cells was shown to be terminated by sialidase treatment, demonstrating dependence on the sialic acid interaction [27]. Interestingly, the interaction of NKp46 with HA on the surface of virus-infected cells is a known mechanism for NK cell recognition and killing of target cells [11], but it appears that the IAV virion itself is able to infect NK cells by internalization of the virion through the same interaction with NKp46 [27].

Human immunodeficiency virus 1 (HIV-1) has also been shown to infect human NK cells both in vitro [36] and in vivo [37]. HIV-1 is an enveloped virus that enters cells by binding with its viral envelope glycoprotein gp120 to the CD4 receptor, and to one of the co-receptors C-X-C chemokine receptor 4 (CXCR4) or C-C chemokine receptor 5 (CCR5) [37]. Virus binding to CD4 brings the virion within range of the co-receptors which then facilitate membrane fusion and subsequent release of the viral core into the host cell cytoplasm. Due to abundant expression of co-receptor CXCR4, NK cells are mostly susceptible to CXCR4-tropic strains of HIV-1 [40]. However, CCR5-tropic strains were also found to infect NK cells, after upregulation of CCR5 expression on NK cells [39,40]. CCR5 expression was either induced by stimulation of NK cells with feeder cells in presence of IL-2 [39], or by
depletion of CCL chemokines that inhibit CCR5 expression [40]. The CD4 receptor is not commonly expressed within the NK lineage, but infection with human herpesvirus 6 (HHV-6, family Herpesviridae) can upregulate CD4 expression in NK cells [23]. It is thought that this HHV-6 infection primes NK cells for HIV infection [23]. Alternatively, a CD4-independent pathway may facilitate HIV-1 entry in absence of CD4 [36].

Epstein Barr virus (EBV, family Herpesviridae) is a lymphotrophic virus that is present in a variety of lymphocyte neoplasms including NK cell lymphoma [16], indicating that EBV can infect NK cells in vivo. Moreover, latently infected non-neoplastic NK cells have been detected in patients with primary EBV infection [17]. The entry mechanism for NK cells is under debate as EBV usually infects oropharyngeal epithelial cells and B cells. The EBV envelope glycoprotein gp350/220 interacts with the CD21 receptor on the surface of B cells and subsequent interaction of the viral gp85–gp25–gp42 glycoprotein complex with MHC–II molecules triggers membrane fusion (reviewed in [47]). Whereas NK cells do express MHC–II molecules [48,49], they do not express CD21 on their cell surface. Interestingly, one study indicated the acquisition of the CD21 receptor by NK cells upon interaction with CD21-positive, EBV-infected cells [18]. Others show a CD21-independent mechanism of NK cell infection by EBV [19].

The receptor-mediated entry of IAV, HIV, and EBV into NK cells indicates that NK cells are permissive for infection due to expression or acquisition of virus entry receptors. However, for many other viruses known to infect NK cells, no receptor has been identified yet. Our group recently showed that RSV can also infect NK cells in vitro and that infection is enhanced in the presence of sub-neutralizing concentrations of RSV–specific antibodies [32]. This study proposes that FcyRIIIA, the IgG-receptor expressed on NK cells, mediates binding to NK cells in the presence of virus–specific antibodies [32]. Although to date it has not been described for other viruses, antibody-mediated entry could be exploited by many more viruses as a mechanism to enter NK cells in individuals that carry virus-specific antibodies.

**Cell–cell interaction–mediated viral entry**

NK cells closely interact with infected cells, for example through the interaction between death receptors and their ligands, in order to clear the viral infection. During this interaction, they may acquire membrane
fragments that contain entry receptors for viruses. This transfer of entry receptors between cells could take place via the immunological synapse, or via intercellular transfer of exosomes (reviewed in [44]). In addition, cell–cell interaction may facilitate direct virus entry through the immunological synapse.

The human T cell leukemia virus 1 (HTLV–1, family Retroviridae) is known to require cell–cell interactions for infection, as infected cells produce virtually no cell–free infectious viral particles [41]. In an extensive imaging study by Igakura and colleagues, the HTLV–1 core complexes and genome were shown to accumulate at cell–cell junctions and were subsequently transferred to uninfected cells [42]. This transfer mechanism was not only observed between T cells, the main target for HTLV, but also between CD4+ T cells and NK cells [42]. Earlier studies had also shown transfer of HTLV–1 from T cells to NK cells, but only after activation of the NK cells with an anti–FcyRIII antibody [43].

Vaccinia virus (VV, family Poxviridae) was also proposed to spread to NK cells through cell–cell contact [33,34]. This virus is able to enter various cell types through non–specific macropinocytosis [50]. VV has been shown to infect NK cells in vitro, but only after co–culturing with other, infected, cell types [33,34]. It was suggested that NK cells become infected by VV upon interaction with infected target cells [33]. These observations imply that there is a transfer of VV particles through the immunological synapse or via exosomes. It must be noted, however, that acquisition of virus entry receptors by NK cells through cell–cell interactions cannot be ruled out for VV.

Two other cell–associated viruses are herpes simplex virus (HSV, family Herpesviridae) and varicella zoster virus (VZV, family Herpesviridae). HSV was shown to enter NK cells after co–culture with infected fibroblasts [22]. Incubation of NK cells with cell–free HSV only induced NK cell lysis at MOIs >100. VZV infection of NK cells in vitro was recently shown after co–culture with infected epithelial cells [24]. Also cell–free direct infection was observed in the case of VZV, indicating that multiple entry mechanisms may be involved.

Although some of these viruses are known to produce virtually no cell–free infectious viral particles, an indirect effect of other infected cell types cannot be ruled out. Infected cells may secrete multiple soluble mediators (e.g. cytokines and chemokines) that could prime NK cells and make them
more prone to infection. Significantly higher NK cell infection rates are seen after stimulation with IL-2 for VZV, and after stimulation with IL-15 for RSV, compared to unstimulated cells [24,32] [unpublished observations]. In conclusion, additional experiments are needed to establish whether infection through co-culture of infected cells with NK cells is a direct or indirect effect.

**Unknown internalization mechanism**

There are many more viruses found to infect NK cells, but research into their entry mechanisms is lacking. DNA of the asymptomatic Torque teno virus (TTV, family Anelloviridae), also known as the transfusion-transmitted virus, has been found in NK cells of viremic individuals [13]. RNA from human pegivirus (HPgV), a virus of the Flaviviridae family formerly known as hepatitis G virus, was detected in NK cells from infected individuals [14]. Furthermore, vesicular stomatitis virus (VSV, family Rhabdoviridae) [35], measles virus (MV, family Paramyxoviridae) [30], human cytomegalovirus (CMV, family Herpesviridae) [15], and HHV–6 [23] have been reported to infect NK cells *in vitro*, but their entry mechanisms are unknown. Entry in the absence of a virus entry receptor may be explained by macropinocytosis, as no specific entry receptor is required for this process [12]. Respiratory syncytial virus (RSV) has been shown to enter epithelial cells through macropinocytosis [31]. Whether entry of NK cells by RSV is mediated through macropinocytosis has not been investigated. In summary, these studies indicate that viral infection of NK cells is not a rare phenomenon but widespread across various virus families.

**MODULATION OF NK CELL FUNCTION AND PHENOTYPE**

Because of the prominent role of NK cells in antiviral immunity, viruses have evolved multiple evasion strategies that affect NK cell effector functions and phenotype. Both these aspects are addressed below and are summarized in Table 1.

**Influence on effector function**

Inhibition of cytotoxicity is the most prominent effect caused by viral infection of NK cells. *In vitro* infection by IAV results in decreased cytotoxic function and induction of apoptosis in both adult and cord blood NK cells.
[25,26,28]. A follow-up study demonstrated that interaction with either the intact influenza virion or free HA protein inhibits NK cell cytotoxicity in a dose-dependent manner through downregulation of the NKp46 and NKp30 signaling pathway [27]. Besides human IAV, avian IAV virions or free avian HA protein also inhibit human NK cell cytotoxicity [29]. Moreover, infection with avian IAV induces apoptosis of NK cells [29]. Thus, these studies demonstrate that binding of IAV is enough to inhibit cytotoxicity, whereas NK cell infection leads to depletion of NK cells through apoptosis. This cellular depletion is supported by evidence of decreased NK cell numbers in both the peripheral blood and lungs of IAV-infected patients [51,52].

Similar effects were demonstrated for VV infection, which also leads to decreased cytotoxicity [33]. However, incubation with UV-inactivated VV did not affect cytotoxicity, indicating that VV replication is required for inhibition [33]. This decreased cytotoxicity was a result of increased NK cell sensitivity to inhibitory killer immunoglobulin-like receptor (KIR) signaling, induced by active viral replication. In addition, decreased perforin secretion was shown for RSV-infected compared to uninfected adult NK cells [32]. The use of a fusion-inhibitor in this study indicated that the mere binding or presence of the virus was not enough to inhibit perforin secretion.

Decreased ability to lyse target cells has also been reported for HSV and MV-infected NK cells [22,30]. The authors did not investigate the effect of non-infectious viral particles, therefore it is unclear whether infection of NK cells was required to inhibit cytotoxicity. For HIV, only a limited effect on the cytotoxic response in vitro was observed in two independent studies [36,40]. The authors propose that the modest impact on killing capacity in vitro was due to the low infection levels that were established [36,40], as depressed NK cell cytotoxicity has been detected in patients with AIDS [53–56]. In addition, viruses can affect NK cell function indirectly by inducing either proliferation or apoptosis. HTLV and HPgV induce increased proliferation and prolonged survival [43,57], whereas in many other in vitro infections apoptosis is induced, as seen for HIV [36], HHV-6 [23], and IAV [25]. Depletion of NK cells during viral infections has been shown in clinical studies for IAV [51,52] and RSV [51,58], but whether this is due to direct infection of NK cells is unknown.

Besides their cytotoxic potential, NK cells are also prominent cytokine and chemokine secreters. Multiple viruses have been shown to affect
cytokine secretion. In the presence of IAV, a downregulation was observed for the production of multiple pro-inflammatory cytokines and chemokines including IFN-γ, GM-CSF, MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) [26]. On the contrary, another study showed an increase in IFN-γ and TNF-α in the cell supernatant after in vitro IAV infection, although this increase was mainly dependent on the presence of IL-15 [28]. In addition, it must be noted that no distinction was made between IAV-infected and IAV-exposed NK cells, and differences in infection levels and the influenza strain used could potentially explain the difference between these studies.

Besides IAV, there are more viruses that increase or suppress NK cell IFN-γ production. RSV-infected NK cells have an increased IFN-γ production, compared to RSV-exposed uninfected or mock-infected NK cells [32]. The combination with decreased perforin secretion of RSV-infected NK cells suggests that RSV infection of NK cells induces a proinflammatory rather than a cytotoxic response that could contribute to the immunopathology seen in vivo. Conversely, incubation of NK cells with HPgV seems to decrease IFN-γ production, but this effect is independent of infection [57].

Taken together, it seems that viruses have evolved multiple mechanisms to inhibit NK cell cytotoxicity or induce apoptosis, which could serve as an important immune evasion strategy. Additionally, the effect of viruses on effector cytokine production by NK cells can vary per virus, leading to variable polarization of the NK cells and (ultimately) in skewed steering of other immune cells.

**Influence on phenotype**

Virus infection does not necessarily modulate NK cell effector functions directly, but often causes changes in receptor expression. As the balance in activating and inhibiting receptor signaling is crucial for NK cell activity, these phenotypical changes may indirectly influence effector functions. Additionally, the phenotype of infected NK cells can be altered by changes in morphology or even transformation into malignant cells.

Downregulation of the expression of activating NK cell receptors has been described for RSV [32]. Independent of infection, the presence of RSV or RSV-antibody complexes results in lower NKG2D and Nkp44 expression levels. In addition, upon infection of NK cells, the expression of (inhibitory) KIRs (KIR3DL1 and KIR2DL2/L3/S2) is upregulated [32]. Functional data
in this study show upregulated IFN-γ production and decreased perforin secretion, but whether this is a direct consequence of the phenotypical changes has not been investigated [32]. Downregulation of activating NK cell receptor NKp46 has been described after IAV exposure of adult and cord blood NK cells [28]. This NKp46 downregulation was accompanied by increased IFN-γ secretion, decreased perforin expression and decreased cytotoxicity [28]. However, a direct link between phenotype and functionality was not investigated. In contrast to these findings, a study performed by Mao and colleagues did not show altered NKp46 expression on NK cells after exposure to IAV virions [27]. An additional study reported no differences in activating (NCR1, NKG2D, NKRPIc, CD244c and Ly49D) or inhibiting (NKG2A, Ly49A, Ly49C/I, and Ly49G2) receptor expression after IAV infection of NK cells, but NKp46 expression was not investigated [26]. Although no difference in receptor expression was found, the cytotoxic potential of NK cells was downregulated in both these studies [26,27]. Incubation of NK cells with VZV leads to decreased CD56 and FcγRIII expression, in combination with an increased expression of CD57, which is a hallmark of NK cell maturity [24]. In addition, VZV induces the expression of skin-homing chemokine receptors CCR4 and cutaneous lymphocyte antigen (CLA) on infected NK cells, thereby promoting a skin-homing phenotype [24]. Data supporting a functional effect of these phenotypical changes is lacking. Viral infection of NK cells has also been shown to cause de novo expression of receptors that are not intrinsically expressed within the NK lineage. HHV-6 induces CD4 expression in infected NK cells, enabling subsequent (CD4-dependent) HIV infection [23]. The effect of CD4 expression on functionality of the HHV-6–infected NK cells was not investigated.

Changes to the morphology of NK cells have been reported following EBV infection in vitro, where infection of NK cells caused cell deformation and increased size [19]. Moreover, EBV infection in vivo has been linked to NK cell malignancies [20,21], suggesting the involvement of EBV in transformation of NK cells.

These studies indicate that each virus induces a different phenotype, often skewed towards an NK cell state that promotes the establishment of a successful infection.
CONTRIBUTION TO VIRAL LOAD

Replication of the viral genome, transcription of viral proteins, and subsequent release of new infectious virions are necessary for virus spread. Since NK cells are infected, they may also contribute to the viral load. A significant role for production of virus particles within NK cells has not been described for the majority of viruses mentioned in this review. Nevertheless, in vitro studies have observed (limited) production of infectious viral particles by NK cells infected with HIV-1 [38, 40], VZV [24], and VSV [35]. HIV-1-infected NK cells produce infectious HIV in vitro and depletion of NK cells from PBMC results in significant reduction of HIV-1 propagation [38,40]. VZV–infected NK cells were able to transmit infectious virus to epithelial cells, suggesting productive infection by VZV [24]. Production of infectious VSV particles has only been investigated in NK cell lines, where VSV infection seemed to result in a persistent infection that continued to produce infectious VSV for over a year [35]. Taken together, it is currently unknown whether infected NK cells serve as a reservoir for certain viral infections and contribute significantly to the dissemination of the virus. Although HIV-1, VZV and VSV seem to establish productive infections, it remains to be investigated whether the amount of released infectious viral particles is enough to significantly contribute to the viral load in vivo.

CONCLUSION AND FUTURE PERSPECTIVES

Accumulating evidence demonstrates that NK cells can serve as target cells for viral infections. Viruses have evolved multiple mechanisms to enter NK cells, change the cellular effector function and affect the subsequent immune response. Literature discussed in this review shows that viruses from nine different families can infect NK cells, indicating this is not an uncommon phenomenon. An overview of the most thoroughly studied viruses that infect NK cells and their effect on NK cell function is shown in Figure 1.

To date, almost all viruses that have been found to infect NK cells are enveloped viruses, with the exception of TTV. This suggests that there is a bigger (evolutionary) advantage for enveloped viruses compared to naked viruses to infect NK cells and modulate anti–viral NK cell immunity.
FIGURE 1  Schematic representation of the most-studied viruses that infect NK cells.

**Abbreviations:** BV, Epstein Barr virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-lymphotropic virus; IAV, influenza A virus; RSV, respiratory syncytial virus; VV, vaccinia virus; VZV, varicella zoster virus.
Cells infected with enveloped viruses display viral proteins on their membrane, making them prone to ADCC by NK cells [4]. Therefore, the inhibition of NK cell ADCC capacity may be specifically of importance as immune evasion strategy for enveloped viruses.

It is apparent that viruses that cause systemic infections will encounter NK cells during an infection. However, infections with viruses like influenza and RSV are restricted to the lungs. Consistent with their important immunological role NK cells are distributed throughout the whole body, including the lungs [59]. Moreover, infection with influenza and RSV has been shown to lead to a pulmonary influx of NK cells in vivo [60–63]. This co-localization of NK cells and viruses during infection supports the potential benefit for viruses to evade the anti-viral NK cells response.

Generally, only a proportion of NK cells is infected in in vitro experiments. For viruses that enter NK cells through target cell interactions, the proximity of NK cells with infected cells seem to be the main determinant for infection. In the case of receptor-mediated entry, limited expression of specific entry receptors may result in non-homogeneous infection of NK cells, although other factors or phenotypic alterations may also be responsible. As NK cells are among the first cells to react to viral infections, inhibition or skewing of a small subset may already have a significant impact on the subsequent immune response. This is supported by increasing evidence showing that NK cells have an important role in shaping the adaptive immune response to viral infections (as reviewed in [64,65]). This also has implications for vaccine development, as attenuated viruses used for vaccination may not infect NK cells and therefore induce different skewing of the adaptive (memory) immune response compared to natural infection. With the limited data that is available, it is impossible to determine whether this altered NK cell response would be beneficial or detrimental for protection.

A caveat of multiple studies that investigate NK cell infection is the lack of a clear distinction between the effect of infection as opposed to mere interaction. Incubation of NK cells with viral particles can lead to modulation of NK cell function in absence of infection. The mere interaction between viral particle and NK cell can be enough to trigger or inhibit certain effects. Whether infection is a prerequisite for NK cell modulation, can be studied using UV-inactivated viruses, as has been done for VV [33], or in the presence of a fusion inhibitor, as has been done for RSV [32]. However, in most of the abovementioned studies these controls were absent.
Several questions remain unresolved and need further investigation. The contribution of infected NK cells to disease severity and viral load remains unclear. Answering this question is challenging, as isolation of (local) cell subsets from patients is often impossible, making it difficult to demonstrate infected NK cells in vivo. The potential use of animal models is limited, as NK cell receptor repertoires differ between species. Moreover, many viruses are host-specific and infection in animals may not mimic the human situation.

It is essential to expand our knowledge on possible cellular targets and reservoirs of viral infections. Intervention strategies to treat viral infections, such as viral entry inhibitors, may have different effects on particular cell types, as was demonstrated for influenza in murine NK cells [45]. Hence, the development of specific therapeutics could benefit from studies into viral infection of different cell types. In addition, viruses that are known to spread through cell–cell interactions may require alternative targets for drug development. The fact that viruses can infect NK cells, suggests that this feature contributes to establishing a successful infection. Therapeutic inhibition of NK cell infection may therefore counteract the evasion strategies of the virus, potentially leading to an enhanced antiviral immune response.
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PART IV

ANTIBODY-DEPENDENT ENHANCEMENT OF DISEASE IN VIVO
PATHOGENESIS OF RESPIRATORY SYNCYTIAL VIRUS INFECTION IN BALB/C MICE DIFFERS BETWEEN INTRATRACHEAL AND INTRANASAL INOCULATION

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ABSTRACT

Human respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract disease requiring hospitalization in infants. There are no market-approved vaccines or antiviral agents available, but a growing number of vaccines and therapeutics are in (pre)clinical stages of development. Reliable animal models are crucial to evaluate new vaccine concepts, but in vivo RSV research is hampered by the lack of well-characterized animal models that faithfully mimic the pathogenesis of RSV infection in humans. Mice are frequently used in RSV infection and vaccination studies. However, differences in the use of mouse strains, RSV subtypes, and methodology often lead to divergent study outcomes. To our knowledge, a comparison between different RSV inoculation methods in mice has not been described in the literature, even though multiple methods are being used across different studies. In this study, we evaluated various pathological and immunological parameters in BALB/c mice after intratracheal or intranasal inoculation with RSV-A2. Our study reveals that intranasal inoculation induces robust pathology and inflammation, whereas this is not the case for intratracheal inoculation. As immunopathology is an important characteristic of RSV disease in infants, these data suggest that in mice intranasal inoculation is a more appropriate method to study RSV infection than intratracheal inoculation. These findings will contribute to the rational experimental design of future in vivo RSV experiments.
INTRODUCTION

Respiratory syncytial virus (RSV) infection is a major cause of severe lower respiratory illness requiring hospitalization in young infants [1]. Currently, there are no market-approved vaccines available, although multiple vaccines are in (pre)clinical stages of development (https://www.path.org/resources/rsv-vaccine-and-mab-snapshot/). Reliable animal models are crucial to evaluate new vaccine concepts. Several animal models have been developed to study RSV infection and disease, including mice, cotton rats, chimpanzees, cattle, and sheep [2,3]. However, none of these models fully replicates the pathogenesis of RSV infection in humans. In light of this, it is pivotal to obtain a thorough understanding of the implications of the use of different methodologies, in order to design experimental approaches that are most suitable for answering specific research questions.

Despite their limitations, mice are the most commonly used animal species for in vivo modeling of RSV disease. Most inbred mouse strains, including the widely used BALB/c and C57 Bl/6 mice, are semi-permissive for RSV infection. These mice need an inoculum with a high viral titer to detect any lower respiratory tract disease symptoms and general measures of illness such as weight loss [4,5]. In addition, the innate and adaptive immune responses after RSV infection differ between humans and mice. For example, mice show a limited recruitment of neutrophils to the lungs, whereas neutrophils are the most abundant cell type during human RSV infection [4,6]. However, the availability of a vast array of mouse-specific reagents and molecular tools makes mice indispensable for gaining mechanistic insights into RSV infection and disease.

Not only is the translation from mouse to human difficult for RSV infection, the comparison between mouse studies is also challenging. This is due in part to differences arising from the use of varying mouse strains and RSV subtypes. The mouse strain is a major determinant of RSV susceptibility and subsequent pathogenesis [7,8]. In addition, responses vary greatly between different RSV subtypes and (clinical) isolates, even if the same mouse strain is used [9,10]. It has also been found that the cell line used for RSV propagation and the purity of the viral inoculum influence infectivity and pathology [11-13]. These reports highlight that appropriate selection of the study setup is crucial to allow
for proper interpretation of the results.

For multiple pathogens other than RSV, it has been suggested that different infection routes also play a role in the inconsistent outcomes between studies. For example, both the site of pathogenesis as well as mortality rates after influenza virus infection in ferrets differed between intranasal and intratracheal inoculation [14,15]. Moreover, different outcomes were observed for intranasal versus intratracheal infection of mice with *Listeria monocytogenes* [16] and *Pseudomonas aeruginosa* [17]. To our knowledge, comparison between these inoculation methods with regard to viral load, pathogenesis, and the immune response has never been undertaken for RSV infection in mice. Although intranasal inoculation methods are most common within the RSV field [18], intratracheal inoculation has also been described [19–21].

In this study, we evaluated virus–induced pathology and the antiviral immune response in BALB/c mice after intratracheal or intranasal inoculation with RSV-A2. Although mice in both groups received an equal dose of the same virus stock, striking differences in viral load, lung damage, and inflammatory mediators were apparent. Our study reveals that at three days post-infection, intranasal inoculation results in robust pathology and immune activation, whereas intratracheal inoculation barely induces either lung damage or inflammation. As immunopathology is an important characteristic of RSV disease in infants, these data suggest that intranasal inoculation in mice is a more appropriate method to mimic the effect of RSV infection in humans than intratracheal inoculation. Together, these findings provide important insights that are essential for the rational design of future *in vivo* experiments with RSV.

**MATERIALS AND METHODS**

**Mice**

Female, specific-pathogen–free BALB/c mice (7–9 weeks old) were obtained from Charles River Laboratories and kept at the animal facilities of Intravacc (Netherlands). The animals were allowed to acclimatize for at least one week. Different batches of mice were used for all five experiments. The study was approved by the Animal Ethical Committee of the Netherlands (CCD; 20185186; April 23, 2018). All experiments were performed in accordance with the guidelines of the institutional animal care committee.
Cells and virus
HEp-2 cells (ATCC CCL-23) were cultured in minimum essential medium (MEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum, and 1% penicillin/streptomycin/glutamine (Gibco). Human RSV-A2 (ATCC, VR1302) was propagated in HEp-2 cells. The virus stock was purified between layers of 10% and 50% sucrose by ultracentrifugation. Virus titer was determined by 50% tissue culture infective dose (TCID50) assay on HEp2 cells. TCID50/ml was calculated using the Spearman and Karber method [22] and converted to plaque-forming units (pfu) per mL by multiplying by 0.69.

Inoculation of mice
Two types of experiments were performed, intratracheal inoculation and intranasal inoculation. The intratracheal inoculation experiment was performed three times, whereas the intranasal inoculation experiment was performed twice. All experiments were carried out on different days. Every experiment consisted of two groups, one RSV- and one mock-infected, of three animals each. The different experiments are indicated by distinct symbols (shape and color) in the graphs.

On day 0, mice were intratracheally or intranasally infected with live, sucrose-purified RSV-A2. Preceding intratracheal inoculation, mice received an intraperitoneal injection of ketamine/xylazine (80 and 10 mg/kg, respectively). Mice were subsequently infected via a cannula in the trachea with 2 x 10^6 pfu RSV-A2 diluted in phosphate buffered saline (PBS) in a total volume of 50 µL. Before intranasal inoculation, mice received anesthesia through inhalation of isoflurane (3.5% in O₂). Mice were then infected with 2 x 10^6 pfu RSV-A2 diluted in PBS in a total volume of 50 µL, which was administered dropwise to the nose. A schematic representation of the inoculation techniques is shown in Figure 1A. Control animals were mock-inoculated either intratracheally or intranasally with 50 µL PBS containing 10% sucrose.

Sample collection
Three days post-infection (dpi), mice were euthanized by exsanguination under isoflurane anesthesia. Bronchoalveolar lavage (BAL) samples and lungs were collected and processed as described below. Weights were measured daily during the course of the experiment.
BAL samples were obtained by infusing 0.8 mL or 0.9 mL (depending on weight of the animal) PBS into the lungs via the trachea, followed by aspiration into a syringe. The BAL sample was centrifuged at 500 × g for 5 min. Cell pellets were collected for flow cytometric analysis and the supernatant was snap-frozen and stored at −80°C for subsequent analysis of cytokine/chemokine and albumin concentrations.

Single-cell suspensions were prepared from mouse lungs by digestion with collagenase and DNase (Sigma-Aldrich, Saint Louis, MO, USA). Cells were pushed through 70 μm cell strainers and erythrocytes were lysed with ammonium–chloride–potassium (ACK) lysis buffer (0.155 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA). Lung cells were used for determination of the viral load.

**Viral load**

The amount of viral RNA was used as a proxy for viral load and was determined by quantitative reverse transcriptase PCR (RT-qPCR). Total RNA was extracted from lung single-cell suspensions using a Nucleospin RNA extraction kit (Machery Nagel, Düren, Germany), and quantified on a Qubit fluorimeter (Invitrogen, Thermo Fisher Scientific). Subsequently, cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) on a thermal cycler (ABI GeneAmp 9700, Applied Biosystems, Thermo Fisher Scientific). Finally, qPCR was performed on a real-time PCR machine (Applied Biosystems StepOne Plus) using SYBR Green reagents (Bio-Rad) and RSV-specific primers. Resulting CT values were converted to arbitrary units (AU) using a standard curve. Amplification with primers corresponding to mouse actin mRNA was performed in parallel and used for normalization. Primer sequences are detailed in Table 1.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>RSV N</td>
<td>Forward: 5′-TGACAGCAGAAGAACTAGAGGC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-TGGGTGATGTGAATTTGCCCT-3′</td>
</tr>
<tr>
<td>Mouse actin</td>
<td>Forward: 5′-CGGTTCGCCATGCCCCCTAGGCTCTCTT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CGTCACACCTTTGATGGAATTGA-3′</td>
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Flow cytometric analysis of immune cell subsets in the BAL
BAL cells were isolated as described above and incubated with anti-mouse CD16/CD32 Fc receptor block (BD Pharmingen, BD Biosciences, San Jose, CA, USA) at 1 µg per 2 x 10^6 cells for 10 min at 4°C. Cells were subsequently incubated with the following antibodies: CD3–Pacific Blue (17A2, Biolegend, San Diego, CA, USA), CD4–APC (RM4–5, BD Pharmingen), CD8a–PE (53–6.7, Biolegend), CD11b–BV711 (M1/70, Biolegend), CD19–BV510 (1D3, BD Horizon, BD Biosciences), CD41–PE-CY7 (MWReg30, Biolegend), CD45–BUV395 (30–F11, BD Horizon), NKp46–BV605 (29A1.4, Biolegend), Ly–6G–PE/Dazzle (1AB, Biolegend). Ultimately, cells were fixed in 3.7% paraformaldehyde in PBS for 10 min before flow cytometric analysis using an LSR Fortessa X20 (BD Biosciences). FlowJo software V10 (FlowJo, BD, Franklin Lakes, NJ, USA) was used for data analysis. The gating strategy for one representative BAL sample is depicted in Supplementary Figure 1.

Albumin and cytokine/chemokine determination in BAL fluid
Albumin concentrations were determined using a mouse albumin ELISA (ICL, Portland, OR, USA), according to the manufacturer’s protocol. Cytokine/chemokine concentrations in BAL samples were analyzed using the mouse anti-virus response panel LEGENDplex (Biolegend), according to the manufacturer’s protocol. This assay contained the following cytokines/chemokines: CCL2 (MCP-1), CCL5 (RANTES), CXCL1, CXCL10 (IP-10), IFN–α, IFN–β, IFN–γ, IL-1β, IL-6, IL-10, IL-12, GM–CSF, and TNF.

Statistical methods
All statistical analyses were performed with Prism 7 software (GraphPad Software, San Diego, CA, USA). Viral load, albumin concentrations, cell counts and cytokine/chemokine concentrations were log transformed before statistical testing. Comparisons between two groups were performed using an unpaired Student’s t-test. Multiple comparisons were analyzed using a one–way analysis of variance (ANOVA), followed by Tukey’s multiple comparisons test. Differences in mouse body weight between mock- and RSV-inoculated animals were analyzed using a two–way ANOVA, using the day and group as variables, followed by Sidak’s multiple comparisons test. P values <0.05 were considered statistically significant.
RESULTS

Intranasal inoculation results in slightly higher viral load and more pronounced pathology compared to intratracheal inoculation

To compare the effect of the inoculation method on viral load and pathology, BALB/c mice were (mock-) infected with vehicle control or RSV-A2 either intratracheally (n = 9 per group, divided over three experiments) or intranasally (n = 6 per group, divided over two experiments). We pooled all data per group for subsequent analysis. Separate experiments are indicated in the graphs by distinct shapes and colors of symbols. The technical differences between intratracheal and intranasal inoculation are illustrated in Figure 1A.

The viral load was determined at three dpi by assessing the amount of viral RNA in total lung cells using RT-qPCR. The results showed a higher

**FIGURE 1** Viral load and pathology following intratracheal or intranasal respiratory syncytial virus (RSV) infection •

Mice were (mock-) inoculated either intratracheally or intranasally with 2 x 10^6 pfu of live RSV-A2 or vehicle control. A. Schematic figure of the intratracheal and intranasal inoculation methods. B–C. Viral load in mouse lung cells as determined by RT-qPCR. Two animals that tested negative for RSV after intratracheal inoculation (B) were excluded from further analysis and removed from panel (C). Geometric mean and SD are depicted. Viral load was log transformed before statistical comparison between the two groups was performed using an unpaired Student’s t-test (*p < 0.05). D. Albumin concentration in the bronchoalveolar lavage (BAL) fluid measured by ELISA. Albumin concentrations were log transformed before analysis by a one-way ANOVA, followed by Tukey’s multiple comparisons test. Geometric mean and SD are depicted. E. Mouse body weight with the weight at day 0 set to 100%. Each data-point represents the mean weight (+/- SD) of all mice from the indicated group: mock intratracheal (IT) (n = 9), RSV IT (n = 7), mock intranasal (IN) (n = 6), RSV IN (n = 6). Body weights between mock- and RSV-infected animals are compared using a two-way ANOVA, followed by Sidak’s multiple comparisons test. Only the IN inoculation groups showed a difference between mock- and RSV-inoculated animals (*p < 0.05). Mice from separate experiments are indicated with a distinct symbol (IT: white / light / black circles; IN: white / dark squares). Abbreviations: AU, arbitrary units; BAL, bronchoalveolar lavage; IN, intranasal; IT, intratracheal; KX, ketamine-xylazine; LRT, lower respiratory tract; RSV, respiratory syncytial virus; SD, standard deviation; URT, upper respiratory tract.
A INTRATRACHEAL INOCULATION
- Direct LRT delivery
- Ix anesthesia

B LUNG VIRAL LOAD

C LUNG VIRAL LOAD

D ALBUMIN IN BAL

E MOUSE BODY WEIGHT

Mock IT (n=9)  Mock IN (n=6)  
RSV IT (n=7)  RSV IN (n=6)
mean lung viral load in intranasally inoculated compared to intratracheally inoculated animals (Figure 1B). Whereas all intranasally infected animals tested positive for viral RNA in the lungs, two animals that had been intratracheally inoculated tested negative for viral RNA. This suggests that intratracheal inoculation is more error-prone than intranasal inoculation, which is likely due to the possibility of inadvertent administration of the inoculum into the esophagus. The two RSV-negative animals were excluded from all further analysis. Upon exclusion of these two animals, the mean viral load was approximately 2-fold higher in the intranasally compared to the intratracheally inoculated animals, which was a statistically significant difference (Figure 1C). No virus was detected in any of the mock-inoculated animals.

To determine whether the high viral load upon intranasal inoculation was accompanied by more pronounced pathology in the lung, we subsequently quantified the albumin concentration in the bronchoalveolar lavage (BAL) fluid and assessed changes in body weight during the course of the experiment. Leakage of serum albumin from the circulation into the lung lumen can be used as a measure for lung damage [23]. Although intratracheal RSV inoculation did not result in any observable lung damage, with albumin concentrations comparable to those of mock inoculation, intranasal inoculation with RSV resulted in higher albumin concentrations compared to both intranasal mock inoculation and intratracheal RSV inoculation, although this was not statistically significant (Figure 1D). In addition to lung damage, animals that had received RSV through intranasal inoculation showed a decrease in body weight, losing almost 5% of body weight at day 1 (Figure 1E). In contrast, animals that had received an intratracheal inoculation did not show a decrease in body weight, but rather gained weight throughout the experiment. Intranasally mock-inoculated animals did not show a change in body weight.

**Virus-induced pulmonary cellular influx is more pronounced upon intranasal compared to intratracheal inoculation**

To investigate the effect of inoculation method on inflammatory parameters, we assessed pulmonary cellular influx by determining (specific) immune cell counts in the BAL at three dpi by flow cytometry. The total leukocyte count in the BAL was significantly increased upon RSV infection compared to that of mock infection for both inoculation methods (Figure 2A).
FIGURE 2  Pulmonary cellular influx following intratracheal or intranasal RSV infection.
Mice were (mock-) inoculated either intratracheally or intranasally with 2 x 10^6 pfu of live RSV-A2 or vehicle control. Cells were isolated from the bronchoalveolar lavage (BAL) and analyzed using flow cytometry. Mice from individual experiments are indicated with a distinct symbol (IT: white / light / dark circles; IN: white / dark squares). Graphs depict absolute cell counts for A. total leukocytes (CD45+), B. NK cells (CD3-, CD56+), C. neutrophils (CD3-, Ly-6G+, CD11b+), D. antigen-presenting cells (CD3-, Ly-6G-, CD11b+), E. CD4 T cells (CD3+, CD4+), and F. CD8 T cells (CD3+, CD8+). All graphs depict geometric mean and SD. Cell count data were log transformed before analysis by a one-way ANOVA, followed by Tukey's multiple comparisons test (*P<0.05, **P<0.01, ***P<0.001). Abbreviations: APCs, antigen-presenting cells.
In addition, total leukocyte counts were significantly higher upon intranasal RSV inoculation compared to those of intratracheal RSV inoculation. Cell type–specific analysis revealed that natural killer (NK) cell (Figure 2B), neutrophil (Figure 2C), antigen–presenting cell (APC) (Figure 2D), and CD4 and CD8 T cell (Figure 2E–F) counts were increased upon RSV infection compared to those of mock infection for both inoculation methods. These differences were statistically significant, except for neutrophils. In addition, NK cell, neutrophil, APC, and CD4 and CD8 T cell counts were markedly higher upon intranasal compared to intratracheal RSV inoculation. These differences were statistically significant, except for CD4 T cells. No increased B cell counts were detected after intratracheal or intranasal RSV inoculation in the BAL at three dpi. These data indicate that the observed pulmonary influx is composed of a variety of immune cells and does not appear to be linked to influx of one particular cell type. Overall, we found that at three dpi the pulmonary cellular influx in the BAL induced by intranasal RSV inoculation was much more pronounced than that induced by intratracheal RSV inoculation.

Elevated virus–induced pulmonary influx upon intranasal inoculation is accompanied by increased cytokine and chemokine levels

The influx of immune cells in the lungs is a hallmark of inflammation and is often accompanied by an increase in the levels of (pro–inflammatory) cytokines and chemokines. To investigate whether intranasal inoculation results in elevated secretion of soluble pro–inflammatory mediators compared to intratracheal inoculation, we determined the concentration of various cytokines and chemokines in the BAL fluid at three dpi. We focused on soluble mediators that are known to be involved in the response against viral infection.

Compared to mock infection, RSV infection via both inoculation methods resulted in increased BAL concentrations of CCL2 (MCP–1), CCL5 (RANTES), CXCL1, CXCL10 (IP10), IFN–α, IFN–γ, and TNF (Figure 3A–G). These differences were statistically significant, except for CCL5 and IFN–α in the case of intratracheal inoculation. IL–1β, IL–12, and GM–CSF showed no difference upon RSV infection compared to mock infection and the
FIGURE 3  Cytokine and chemokine levels in the bronchoalveolar lavage following intratracheal or intranasal RSV infection.·○○○
Mice were (mock-) inoculated either intratracheally or intranasally with 2 x 10⁶ pfu of live RSV-A2 or vehicle control. Bronchoalveolar lavage (BAL) fluid was analyzed at 3 dpi using a multiplex mouse anti-virus response panel immunoassay. A–G. Concentrations in pg/ml of CCL2, CCL5, CXCL1, CXCL10, IFN-α, IFN-γ, and TNF. Mice from individual experiments are indicated with a distinct symbol (IT: white / light / dark circles; IN: white / dark squares). Cytokine concentrations were log transformed before analysis by a one-way ANOVA, followed by Tukey’s multiple comparisons test (**P<0.01, ***P<0.001). H. Concentration of indicated soluble pro-inflammatory mediators after either intratracheal or intranasal RSV infection. The bars represent the geometric mean and SD of all animals belonging to the indicated groups. Fold change between intratracheal and intranasal infection is indicated above each pair of bars. Abbreviations: BAL, bronchoalveolar lavage; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; IN, intranasal; IP10, interferon gamma-induced protein 10; IT, intratracheal; MCP-1, monocyte chemoattractant protein 1; RANTES, Regulated upon Activation, Normal T cell Expressed and presumably Secreted; RSV, respiratory syncytial virus; SD, standard deviation.

levels of IFN-β, IL-6, and IL-10 remained below the limit of detection. Compared to intratracheal RSV inoculation, mice that had been intranasally inoculated with RSV showed 3- to 8-fold higher levels of CCL2, CCL5, CXCL1, CXCL10, IFN-α, IFN-γ, and TNF (Figure 3H). These differences were all statistically significant. Similar to the pulmonary cellular influx, we observed differences in some of the pro-inflammatory mediators between experiments performed on different days. In summary, RSV infection induced expression of a number of pro-inflammatory cytokines and chemokines. Animals that were inoculated intranasally showed markedly higher levels of RSV-induced pro-inflammatory mediators in BAL fluid than intratracheally inoculated animals at three dpi.

DISCUSSION

Seemingly minor methodological differences can have profound effects on the outcome of animal studies. It is therefore of pivotal importance to understand beforehand the implications of choosing a particular method, in order to allow for proper interpretation of the results obtained at the end of the experiment. In the present study, we compared the pathological and immunological effects of RSV infection via intratracheal or intranasal inoculation. The latter resulted in a slightly higher viral load that was accompanied by more pronounced pathology compared to intratracheal
inoculation. In addition, inflammatory parameters such as pulmonary cellular influx and pro-inflammatory cytokine and chemokine levels were significantly higher after intranasal versus intratracheal inoculation.

A possible explanation for the observed differences in viral load, pathology, and host responses is the difference in (primary) infection site between intratracheal and intranasal inoculation. During intratracheal inoculation, the virus is delivered directly to the lung, presumably resulting in an infection that is confined to the lower respiratory tract, at least initially. In contrast, intranasal inoculation with the volume used in this study results in infection of both the upper and lower respiratory tract [24]. It is conceivable that viral replication in the upper airways contributes to a higher viral load in the lungs. In addition, it is likely that the host response to viral infection in the upper airways influences the immune response in the lower airways. Previous studies have shown that viral replication and host responses may vary depending on the anatomical localization of the RSV infection. For example, in vitro studies have shown increased viral production in human nasal epithelial cells compared to bronchial epithelial cells [25]. In contrast, ex vivo studies with primary pediatric epithelial cell cultures have shown lower RSV titers accompanied by slightly lower cytokine responses in nasal cells compared to bronchial cells [26]. Interestingly, influenza virus infection in ferrets resulted in higher mortality rates after intratracheal inoculation compared to intranasal inoculation [15]. These findings highlight that virus-specific information on the outcomes of different inoculation routes is essential for the design of future studies.

Another important factor that might have contributed to the observed differences is the differential use of anesthetics between the two inoculation methods. For intratracheal inoculation, which is a more invasive procedure, the induction of anesthesia through ketamine/xylazine injection is common practice. Intranasal inoculation only requires a short duration of anesthesia and is therefore performed under inhaled isoflurane anesthesia. Although not specifically investigated for RSV infection, the use of anesthetics can have a major impact on the immune system [27-30]. Multiple studies have reported that ketamine possesses anti-inflammatory properties and impairs the release of pro-inflammatory cytokines [29,31,32]. In addition, ketamine has been shown to have antiviral properties against rabies virus [33]. Although isoflurane has also been shown to reduce inflammation, these effects were mostly seen after long-term exposure.
[30,34,35]. Taken together, the use of ketamine/xylazine anesthesia for intratracheal inoculation may, at least in part, explain the observed lower immunopathology compared to intranasal inoculation. Considering this, we found it striking that a considerable number of published studies fail to specify the type of anesthesia that was used during RSV infection in mice. The main limitation of our study is the fact that the intratracheal and intranasal inoculations were performed in separate experiments, which is a suboptimal experimental setup to compare the two methods. However, during each experiment, exactly the same virus dose was used, originating from the same virus batch. In addition, all animals were obtained from the same source, were of similar age, and were handled at the same facility. Nevertheless, we did observe differences between experiments performed on different days, especially regarding the immunological read-outs. As we used female mice for our study, a possible explanation for these effects is the ovarian cycle of the different batches of animals. Multiple studies have shown differences in the immune response between the different ovarian cycle phases [36,37]. For future experiments, the use of male mice might be more appropriate. However, this phenomenon also highlights the importance of performing experiments on multiple days to control for these kinds of variations. Taken together, we believe the experimental conditions of our study were similar enough to warrant a comparison between the two inoculation methods, especially considering the fact that the observed differences are quite striking and may have implications for the choice of inoculation method for future animal experiments.

Another limitation of our study is the fact that most pathological and immunological parameters were only assessed at one time-point post infection. It is possible that the observed differences are due to variations in the kinetics of virus replication and host response depending on the site of initial infection and/or anesthesia. However, the absence of a change in body weight upon intratracheal inoculation suggests this is not the main cause underlying the observed effects. Notably, we only assessed the short-term consequences of viral infection on pathology and host response. It remains a possibility that long-term immune responses, such as humoral and cellular memory responses, are differently affected by the two inoculation methods. Nevertheless, when focusing on these short-term responses, intranasal inoculation appears to be the method of choice.

In conclusion, we have demonstrated striking differences between
intratracheal and intranasal RSV inoculation with regards to pathological and immunological read-outs, which may be explained by differences in the (initial) site of infection or the differential use of anesthetics. Based on our findings, intranasal inoculation appears to be better suited to the study of RSV-induced immunopathology than intratracheal inoculation. Not only does intranasal inoculation more accurately mimic the natural route of infection, it is also a less invasive procedure, requires milder anesthetics, is less error-prone, and, as we have demonstrated, induces more pronounced pathology and immune responses than intratracheal inoculation. Although it remains to be investigated whether the route of administration or the anesthetic is the main factor underlying the observed differences, it is evident that the selection of inoculation method (even when both techniques deliver the virus to the lower respiratory tract) is of crucial importance to obtain the most appropriate model to mimic RSV disease in humans.

Supplementary Materials are available online at:
https://www.mdpi.com/1999-4915/11/6/508#supplementary
REFERENCES

IN VIVO INDICATIONS FOR ANTIBODY-DEPENDENT ENHANCEMENT OF RSV INFECTION AND DISEASE

Manuscript in preparation

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Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract infections in infants. Hospitalization peaks below six months of age, when maternal antibody levels are declining. Although it is generally assumed that antibodies hold the key to protection against severe RSV disease, it has also been suggested that sub-neutralizing antibody titers lead to enhanced RSV infection and disease. However, in vivo data supporting this hypothesis of antibody-dependent enhancement (ADE) is lacking.

In this in vivo study, we evaluated the viral load, pathology and inflammatory response in the presence of serially diluted RSV-specific antibodies, mimicking the waning maternal antibody titer in infants. In contrast to the expected reduction in viral load observed at high antibody concentrations at 3 days post infection, low antibody concentrations resulted in a significantly higher viral load compared to the absence of antibodies. Moreover, mice that received low antibody concentrations displayed slightly increased pathology compared to the absence of antibodies. Inflammatory markers, such as pulmonary cellular influx and some cytokine and chemokine concentrations, were marginally increased in the presence compared to the absence of antibodies.

These data provide the first clues towards the occurrence of enhanced RSV infection and disease in vivo, mediated by antibodies induced by natural RSV infection, indicating that a sub-protective humoral response may have pathological consequences. Additional experiments should be performed to further elucidate this phenomenon, which, if substantiated, would be an important point of concern in view of the development of (maternal) RSV vaccines.
INTRODUCTION

Respiratory syncytial virus (RSV) infection is a major cause of severe respiratory illness requiring hospitalization in young infants [1]. Hospitalization for severe RSV-mediated disease peaks between six weeks and six months of life [2,3]. Maternal antibodies are present during these first months of life, but levels are rapidly waning over time [4–6]. Multiple studies show that high maternal antibody titers are associated with protection against RSV infection or disease [6–9]. However, others demonstrate that high titers do not have a protective effect [10–13] or even associate with an increased risk of recurrent wheezing [14].

Originally, the main functionality of antibodies was thought to be direct virus neutralization. However, in addition to reducing the infectivity of the virus, antibodies may influence the immune response through the formation of immune complexes and opsonization of virus–infected cells. There is increasing evidence that antibody effector functions besides neutralization play an important role in RSV infection and disease (reviewed in [15,16]). In contrast to their beneficial role in providing protection against infection and disease, antibodies have also been implicated in enhancement of disease. Interestingly, the disastrous vaccine trials in the 1960s with a formalin-inactivated (FI) RSV vaccine induced poorly-neutralizing antibodies which have been suggested to be involved in vaccine–enhanced disease upon natural infection [17,18].

The presence of RSV–antibody complexes in the lungs during severe RSV infection has been demonstrated in multiple studies, but their role in infection and disease remains largely unknown [17,19,20]. FI–RSV vaccine–induced antibodies contributed to enhanced disease upon subsequent natural infection in a mouse model [17]. However, in natural RSV infection, the contribution of RSV–antibody complexes in disease is debated. In hospitalized RSV–infected infants, immune complexes were identified in all groups independent of the severity of disease [20]. In an RSV infection mouse model, RSV–antibody complexes were shown to initiate the adaptive immune response [21]. In addition, immune complexes have been shown to lead to increased infection levels in Fc gamma receptor (FcyR)–expressing (primary) cells in vitro [22–26]. However, thus far no evidence for enhancement of infection or disease by naturally occurring antibodies has been shown during RSV infection in vivo.
In utero, every infant receives RSV-specific antibodies from the mother via transplacental transfer. Maternal vaccination to boost these antibodies is considered for the prevention of RSV disease. It is therefore essential to understand the implications of the presence of these maternal RSV-specific antibodies, and the waning thereof after birth, on the immune response during subsequent RSV infection in vivo. However, assessment of the RSV-specific immunological response is routinely performed in naïve animals. Although the absence of antibodies makes the interpretation of the results of such studies less complex, it only provides a limited view of the processes underlying severe disease in infants. Mimicking the situation present in the target population is essential, especially in the context of assessing therapeutic or prophylactic treatments.

In this study, we infected BALB/c mice with RSV-A2 after administration of serially diluted RSV-specific antibodies, mimicking the waning maternal antibody levels in infants. Subsequently, we evaluated the effect of different antibody concentrations on viral load, pathology, and inflammatory response. Although preliminary, our study provides the first indications that sub-neutralizing concentrations of naturally occurring antibodies may indeed enhance RSV infection and disease. Further research is necessary to confirm these findings and establish the underlying mechanisms. This knowledge will be key for the development of safe and effective (maternal) RSV vaccines.

METHODS

RSV-A2 production
HEp-2 cells (ATCC CCL-23) were cultured in Minimum Essential Medium (MEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum, and 1% penicillin/streptomycin/glutamine (Gibco). Human RSV-A2 (ATCC, VR1302) was propagated in HEp-2 cells. Virus stocks were purified between layers of 10% and 50% sucrose by ultracentrifugation. Virus titers were determined by 50% tissue culture infective dose (TCID50) assay on HEp2 cells. TCID50/ml was calculated using the Spearman and Karber method [27] and converted to plaque-forming units (pfu) per ml by multiplication with 0.69.
Passive transfer experiment in mice
Female, specific-pathogen-free BALB/c mice (7-9 weeks old) were obtained from Charles River Laboratories and kept at the animal facilities of Intravacc (Netherlands). The animals were allowed to acclimatize to their new environment for at least one week. The study was approved by the Animal Ethical Committee of the Netherlands (CCD). All experiments were performed in accordance with the guidelines of the institutional animal care committee.

The study included 6 groups of 6 animals each, divided over 2 experiments that were performed on separate days (indicated by distinct colors in each graph). On day 0, mice first received an intravenous (IV) injection of 200 \( \mu \)l antibodies or vehicle control (PBS). Three hours after IV injection, mice were anesthetized using inhaled isoflurane before intranasal inoculation with 2 x 10^6 pfu of live RSV-A2 in 50 \( \mu \)L PBS. Control animals received intranasal mock inoculation with 50 \( \mu \)L PBS containing 10% sucrose. One group was mock-infected after receiving an IV injection with the vehicle control. The other groups were RSV-infected after receiving an IV injection of vehicle control, 100, 10, or 1 mg/ml intravenous immunoglobulins (IVIg, Baxter), or 2 mg/ml Palivizumab (Synagis, Alliance Healthcare).

Sample collection
At 3 days post-infection (dpi), anesthesia was induced by isoflurane and mice were euthanized by exsanguination. Bronchoalveolar lavage (BAL) specimens, lungs, and blood were collected and processed as described below.

BAL specimens were obtained by infusing 0.8 mL or 0.9 mL (depending on weight of the animal) phosphate buffered saline (PBS) into the lungs via the trachea, followed by aspiration into a syringe. The BAL fluid was centrifuged at 500 \( \times \) g for 5 min. Cell pellets were collected for flow cytometry analysis and the supernatant was snap-frozen and stored at -80°C for subsequent analysis of antibody concentrations, cytokine levels and albumin concentrations.

Single-cell suspensions were prepared from mouse lungs by digestion with collagenase and DNase (Sigma–Aldrich). Cells were pushed through 70 \( \mu \)M cell strainers and erythrocytes were lysed with ammonium-chloride–potassium (ACK) lysis buffer (0.155 M NH_4Cl, 10 mM KHCO_3, and 0.1 mM Na_2EDTA). Lung cells were used for determination of the viral load.
Blood was collected in heparin collection tubes. Collection tubes were centrifuged at 750 × g for 10 min after which plasma was harvested. Plasma was stored at -80°C until measurement of antibody concentrations.

**Viral load**

Viral RNA was quantified as a proxy for viral load by quantitative reverse transcriptase PCR (RT-qPCR). Total RNA was extracted from lung single-cell suspensions using a Nucleospin RNA extraction kit (Machery Nagel), and quantified on a fluorimeter (Qubit). Subsequently, cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad) on a thermal cycler (Applied Biosystems PCR system 9700). Quantification was performed on a real-time PCR machine (Applied Biosystems StepOne Plus) using SYBR green reagents (Bio-Rad) and RSV-specific primers. Resulting $C_T$ values were converted to arbitrary units (AU) using a standard curve. Amplification with primers corresponding to mouse actin mRNA was performed in parallel and used for normalization. Primer sequences are detailed in Table 1.

**Plasma and BAL antibody levels**

To quantify the concentration of RSV-specific IgG in plasma and BAL samples at 3 days post infection, a multiplex immunoassay was performed as described before [28]. In short, plasma and BAL samples were incubated with RSV antigen-coupled beads and the bound antibodies were labeled with a secondary goat anti-human IgG antibody (Jackson Immunoresearch Laboratories). Measurement of the samples was performed on a Bio-Plex 200 in combination with Bio-Plex Manager software version 6.1 (Bio-Rad). For each analyte, median fluorescence intensity (MFI) was converted to arbitrary units/ml (AU/ml) by interpolation from a 5-parameter logistic

<table>
<thead>
<tr>
<th>Transcrip</th>
<th>Sequence</th>
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<tr>
<td>RSV N</td>
<td>Forward: 5’-TGACACGACAGAAGAACTAGAGGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TGGGTGATGTGAATTTGCCCT-3’</td>
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<tr>
<td>Mouse actin</td>
<td>Forward: 5’-CGGTTCGGATGCCCTGAGGCTTT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTCACACTTCATGATGGAATTGA-3’</td>
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standard curve from an in-house reference serum pool known to have high RSV titers [28].

**Flow cytometric analysis of BAL cell subsets**

BAL cells were isolated as described above and incubated with anti-mouse CD16/CD32 Fc receptor block (BD Pharmingen) at 1 µg per 2 x 10⁶ cells for 10 min at 4°C. Cells were subsequently incubated with the following antibodies: CD3-Pacific Blue (17A2, Biolegend), CD4-APC (RM4-5, BD Pharmingen), CD8a-PE (53–6.7, Biolegend), CD11b-BV711 (M1/70, Biolegend), CD19-BV510 (1D3, BD Horizon), CD41-PE-CY7 (MWReg30, Biolegend), CD45–BUV395 (30–F11, BD Horizon), NKp46–BV605 (29A1.4, Biolegend), and Ly–6G–PE/Dazzle (1AB, Biolegend). Ultimately, cells were fixed in 3.7% paraformaldehyde in PBS for 10 minutes before flow cytometric analysis using an LSR Fortessa X20 (BD Biosciences). FlowJo software V10 (FlowJo, LLC) was used for data analysis. Gating strategy for one representative BAL sample is depicted in Supplementary Figure 1.

**Albumin and cytokine/chemokine determination in BAL fluid**

Albumin concentrations were determined using a mouse albumin ELISA (Immunology Consultants Laboratory), according to the manufacturer’s protocol. Cytokine concentrations in BAL samples were analyzed using the mouse anti-virus response panel LEGENDplex (Biolegend), according to the manufacturer’s protocol. Only cytokines and chemokines that consistently had levels above the detection threshold are depicted.

**Statistical methods**

All statistical analyses were performed with Prism 7 software (GraphPad). Viral load, cell counts, albumin concentrations and cytokine/chemokine concentrations were log transformed before statistical testing. Multiple comparisons were analyzed using a one-way ANOVA, followed by Dunnett’s multiple comparisons test. P values <0.05 were considered statistically significant.
RESULTS

Passive transfer of IVIg is detectable in both plasma and lungs
To mimic infants’ waning maternal antibody levels in the absence of a cellular memory response, we passively transferred serially diluted IVIg (containing naturally induced RSV–specific antibodies) to RSV–naive mice. As we injected 200 µL per animal, we expect the group that received 100 mg/mL to obtain an IVIg plasma concentration similar to healthy human adults, which is approximately 10 mg/mL. The other two concentrations represent the waning (maternal) antibody titers seen in infants. Subsequently, mice were challenged with RSV–A2 and sacrificed at day 3 after infection. The presence of IgG in blood plasma and BAL was assessed with an RSV antigen–specific MIA. Antibodies were readily detectable at 3 days after injection for the different RSV antigens: the attachment (G) protein from both RSV–A and RSV–B strains (Figure 1A–B), the post– and pre–fusion conformation of the fusion (F) protein (Figure 1C–D), and the nucleoprotein (N) (Figure 1E). The concentration of pre–F, post–F and N specific antibodies was the highest, whereas G–specific antibody levels were low, as described in literature [9,28]. To confirm that IV injection of IVIg results in transfer of RSV–specific antibodies to the site of infection, BAL samples obtained at 3 days after administration were also assessed. RSV–specific IgG was detectable in the BAL fluid at 100–fold lower concentration than in plasma, as exemplified for RSV pre–F– and G–specific antibodies administered at 100 mg/ml IVIg concentration (Figure 1F).

FIGURE 1 Passive transfer of IVIg is detectable in blood & lungs
Mice were intravenously injected with 200 µL of the indicated doses of IVIg or 2 mg/mL Palivizumab. Subsequently, mice received intranasal inoculation with 2 x 10^6 pfu of live RSV–A2 or PBS containing 10% sucrose (mock). Mice from separate experiments are indicated with a distinct color. A–E. Plasma IgG levels were determined by multiplex immunoassay for the attachment protein (G) from both RSV–A and –B strains (A–B), the post– and pre–fusion conformation of the fusion (F) protein (C–D), and the nucleoprotein (N) (E). (F) Comparison of plasma and bronchoalveolar lavage IgG levels from animals that received 100 mg/mL IVIg or 2 mg/mL Palivizumab. Each dot represents six animals. All graphs depict geometric mean and SD. Abbreviations: AU, arbitrary units; BAL, bronchoalveolar lavage; IgG, immunoglobulin G; IVIg, intravenous immunoglobulin; RSV, respiratory syncytial virus.
**Antibody-Dependent Enhancement in Vivo**

**Plasma B ALL**

<table>
<thead>
<tr>
<th>IVIg (mg/mL)</th>
<th>RSVMock</th>
<th>RSVMock</th>
<th>Palivizumab</th>
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<tr>
<td>0</td>
<td>0.1</td>
<td>1</td>
<td>10</td>
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**RSV-specific Abs (AU/mL)**

- G-specific IgG (RSV-A)
- G-specific IgG (RSV-B)
- N-specific IgG
- Pre-F-specific IgG (RSV-A)
- Post-F-specific IgG (RSV-A)

**G-specific IgG (RSV-B)**

**N-specific IgG**
Currently, the only available prophylactic treatment against RSV infection is the administration of an F–specific monoclonal antibody (Palivizumab), which has been shown to reduce viral load in in vivo mouse models [29,30]. Therefore, we included a group receiving this antibody in our experiments and measured antibody levels in both plasma and BAL. The plasma and BAL samples of these mice showed a pre- and post–F–specific signal as Palivizumab binds both F protein conformations (Figure 1A–F).

Sub-neutralizing antibody concentrations increase viral load and pathology
A reduction in viral load was observed for animals that received high doses of IVIg (100 mg/mL or 10 mg/mL) or Palivizumab (Figure 2A). This decrease was only statistically significant in mice that received the highest dose of IVIg or Palivizumab. In contrast, mice that received IVIg at 1 mg/mL showed a statistically significant increase in viral load at 3 dpi compared to control animals that were infected in the absence of antibodies. Although the viral load showed considerable variation in this group, it was consistently higher than the viral load detected in the RSV-infected group that had not received antibodies.

**FIGURE 2** Sub-neutralizing antibody concentrations enhance viral load & pathology
Mice were intravenously injected with 200 µL of the indicated doses of IVIg or 2 mg/mL Palivizumab. Subsequently, mice received intranasal inoculation with 2 x 10^6 pfu of live RSV–A2 or PBS containing 10% sucrose (mock). Mice from separate experiments are indicated with a distinct color. A. Viral load in mouse lung cells as determined by RT–qPCR. Geometric mean and SD are depicted. Viral load was log transformed before analysis by a one–way ANOVA, followed by Dunnett’s multiple comparisons test (*P<0.05, **P<0.01, ***P<0.001). The dotted line indicates the geometric mean of the RSV–infected control animals that received no antibodies. B. Mouse body weight with the weight at day 0 set to 100%. Each dot represents the mean weight (+/− SD) of the mice from both experiments within one group (n = 6). C. Albumin concentration in the BAL fluid was measured by ELISA. Albumin concentrations were log transformed before analysis by a one–way ANOVA, followed by Dunnett’s multiple comparisons test. Geometric mean and SD are depicted. The dotted line indicates the geometric mean of the RSV–infected control animals that received no antibodies. **Abbreviations:** AU, arbitrary units; BAL, bronchoalveolar lavage; IgG, immunoglobulin G; IVIg, intravenous immunoglobulin; Pali, palivizumab; RSV, respiratory syncytial virus; RT–qPCR, quantitative reverse transcriptase polymerase chain reaction.
ANTIBODY-DEPENDENT ENHANCEMENT IN VIVO

**A**  Viral load

```
0 0 1 0 0 1 0 1 P ali
0
1 0 0
2 0 0
3 0 0
4 0 0
5 0 0
```

**B**  Mouse body weight

```
0 mg/mL IV Ig
0 mg/mL IV Ig
100 mg/mL IV Ig
10 mg/mL IV Ig
1 mg/mL IV Ig
Pali
```

**C**  Albumin in BAL
To investigate whether the enhanced viral load resulted in enhanced pathology, we assessed body weight loss and albumin concentrations in the BAL as a measure for lung damage. Mock animals showed no decrease in weight loss, whereas all mice that were RSV-infected showed weight loss at 1 dpi (Figure 2B). The weight loss was moderate for animals that had received no antibodies, a high concentration of IVIg, or Palivizumab. The most pronounced decline in weight was seen for the mice that had received the lowest IVIg concentrations (10 mg/mL and 1 mg/mL). In addition, a slightly higher mean albumin concentration was measured in mice that had received 1 mg/mL IVIg, but this was not statistically significant (Figure 2C). Taken together, viral load was higher in mice that received low concentrations of antibodies compared to the absence of antibodies. This higher viral load was accompanied by a trend towards more body weight loss and lung damage.

Antibodies have only minor effects on lung inflammation at 3 dpi

The immune response is considered to be an important contributor to RSV-induced pathology in humans. Therefore, we next investigated potential differences in immunological parameters between mice in the presence or absence of antibodies. Both the pulmonary cellular influx and the secretion of cytokines and chemokines in the BAL were assessed.

The total influx of leukocytes in the BAL was significantly increased in all RSV-infected compared to mock-infected mice (Figure 3A).
ANTIBODY-DEPENDENT ENHANCEMENT IN VIVO

A) Total influx

B) NK cells

C) Neutrophils

D) CD11b(+) APCs

E) CD4(+) T cells

F) CD8(+) T cells

Legend:

- Mock
- RSV
- PAL

Significance:

- *** p < 0.001

*Note: The graphs show the counts of various cell types in BAL fluid (Bronchoalveolar Lavage) at different IVIg concentrations (mg/mL) for RSV and Mock groups. The data is presented as mean ± standard error of the mean (SEM).
In addition, mice that had received IVIg appeared to have slightly higher pulmonary leukocyte influx than mice that had received no antibodies or Palivizumab, although this difference was not statistically significant. This shows that the pulmonary leukocyte influx is independent of the viral load, considering that the viral load was significantly reduced in animals that had received a high dose of IVIg.

To specify whether there were differences in specific cell subsets, we investigated the composition of the pulmonary leukocyte influx. The numbers of natural killer (NK) cells (Figure 3B), neutrophils (Figure 3C), antigen-presenting cells (APCs) (Figure 3D), and CD4$^+$ or CD8$^+$ T cells (Figure 3E-F) were all significantly increased upon RSV infection compared to mock infection at 3 dpi. No significant increase in B cell numbers upon RSV infection was detected at 3 dpi (data not shown). Except for the APCs, which showed similar numbers in the presence or absence of antibodies, all RSV-induced cell types showed a similar pattern as the total leukocyte influx. Of note, the presence of Palivizumab did result in a slightly reduced influx of NK cells, neutrophils, and APCs for some of the animals compared to the control animals that received no antibodies.

Besides the pulmonary leukocyte influx, the presence of soluble mediators is another measure of lung inflammation. We measured the presence of different cytokines and chemokines that are related to the early response against viral infection. A clear response upon RSV infection was measured for CCL2, CCL5, CXCL1, CXCL10, IFN-α, IFN-γ, and TNF.

**FIGURE 4** Antibodies have only minor effects on lung inflammation

Mice were intravenously injected with 200 µL of the indicated doses of IVIg or 2 mg/mL Palivizumab. Subsequently, mice received intranasal inoculation with 2 x 10$^6$ pfu of live RSV-A2 or PBS containing 10% sucrose (mock). Fluid from the bronchoalveolar lavage was analyzed using a multiplex mouse anti-virus response panel. Mice from separate experiments are indicated with a distinct color. A–G. Only cytokines and chemokines that showed a response upon RSV infection are depicted. Cytokine concentrations were log transformed before analysis by a one-way ANOVA, followed by Dunnett’s multiple comparisons test (**P<0.001). Geometric mean and SD are depicted. The dotted line indicates the geometric mean of the RSV-infected control animals that received no antibodies. Abbreviations: BAL, bronchoalveolar lavage; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; IFN, interferon; RSV, respiratory syncytial virus; TNF, tumor necrosis factor.
ANTIBODY-DEPENDENT ENHANCEMENT IN VIVO

**Figure 1**: Summary of cytokine levels in response to RSV infection with different IVIg concentrations.

**A** CCL2

**B** CCL5

**C** CXCL1

**D** CXCL10

**E** IFN-α

**F** IFN-γ

**G** TNF

Legend:
- **Mock**: Control group without RSV infection.
- **RSV**: RSV infected group.
- **IVIg**: Intravenous immunoglobulin.
- **Pali**: Tested group.

**Significance Levels**:
- *******: P < 0.001
- ****: P < 0.01
- ****: P < 0.05

**Cytokine Measurements**:
- **TNF (pg/mL)**
- **IFN-α (pg/mL)**
- **IFN-γ (pg/mL)**
- **CCL2 (pg/mL)**
- **CCL5 (pg/mL)**
- **CXCL1 (pg/mL)**
- **CXCL10 (pg/mL)**
No significant upregulation was seen for IL-12, IL-1β, and GM-CSF and levels of IL-10, IFN-β, and IL-6 were below detection level (data not shown). The mean concentrations of CXCL1, CCL2, CXCL10, TNF, and IFN-α were slightly increased in the presence of IVIg compared to controls, but this difference was not statistically significant. Comparable to the cellular influx in the BAL, some of the animals that received Palivizumab showed decreased CCL2, CCL5 and IFN-γ levels. In summary, these results suggest that both the leukocyte influx and the concentrations of the assessed soluble mediators in the BAL at 3 dpi are independent of the viral load.

**DISCUSSION**

In this study, we evaluated an in vivo mouse model for RSV infection in infants, in which we mimic the waning of transplacentally transferred maternal IgG through the intravenous injection of serially diluted IVIg. Using this in vivo model, we demonstrate that animals that received low concentrations of IVIg show higher RSV viral load and possibly pathology, as measured by a slight increase in weight loss after infection. To our knowledge, this provides the first in vivo indications of enhancement of RSV infection and disease by antibodies induced by natural RSV infection. It must be noted that this has only been investigated at 3 dpi and no clear correlation with inflammation was found. Therefore, follow-up research to corroborate our findings is urgently needed, as this would have major implications for preventive measures such as (maternal) vaccination.

ADE of infection is a well-studied phenomenon in vitro for multiple viruses (as extensively reviewed in [31]). For RSV, both monoclonal antibodies and RSV-immune serum have been shown to induce ADE of infection in vitro in a range of FcγR-expressing (primary) cells [22,23,25,26,32]. However, only for dengue virus, mouse models have demonstrated ADE of infection and ADE of disease in vivo [33-35]. It was shown that the presence of dengue-specific antibodies led to enhanced systemic virus levels and increased disease severity in mice [33]. In addition, enhanced dengue disease was found in the presence of maternally acquired dengue-specific antibodies, indicating a role for maternal antibodies in ADE of dengue virus infection in mice [35]. Recently a large long-term pediatric cohort in Nicaragua supported these mouse studies by demonstrating that...
high dengue-specific antibody titers correlate with protection, whereas intermediate antibody titers correlate with severe dengue disease in humans [36].

In the current study, we use human IVIg in a mouse model. Previous studies have shown that human IgG can bind mouse FcγRs and can induce cellular effector functions in wild-type mice [37,38]. Additionally, it was shown that human IgG interacts with the mouse neonatal Fc receptor, resulting in a similar half-life of human IgG in mice and humans [39]. However, it is debated whether mouse Fc receptors mediate similar effector functions as human Fc receptors [40]. Besides RSV-specific antibodies, IVIg contains antibodies with a broad range of specificities. Although we expect the effect on RSV viral load and pathology to be caused by RSV-specific antibodies, we cannot rule out effects by other antibodies. Efforts to deplete IVIg of RSV-specific antibodies are ongoing.

In accordance with previous studies [41,42], our results show that RSV-specific antibodies are readily transferred from the blood to the lung lumen and can be detected in the BAL, albeit in 100-fold lower concentrations than in plasma. These data highlight that the presence of neutralizing antibody titers in plasma does not guarantee the presence of neutralizing titers in the lungs, which is the primary site of infection for RSV. Therefore, it is essential to realize that measuring the neutralizing capacity of antibodies in the plasma only provides limited information about the local neutralizing response in the lungs.

In our study, we observed a reduction of viral load in the presence of high IVIg concentrations, while pulmonary cellular influx and secretion of inflammatory cytokines and chemokines remained high. This indicates that the inflammatory parameters we measured are independent of viral load. Earlier studies in cotton rats have also shown decreased viral titers in the presence of monoclonal antibodies, accompanied by a lack of decrease in inflammation markers [43,44]. The mechanisms underlying severe RSV disease are unclear, but both heightened viral load [45–47], as well as a pathological excessive immune response [48] are thought to play a role in severe RSV disease. Although many studies exclusively look at decreased viral load for effectiveness of monoclonal antibodies and vaccines, our observations indicate that the immune response should also be monitored. Interestingly, the mice that received Palivizumab show lower pulmonary cellular influx and lower secretion of cytokines and chemokines
compared to the 100 mg/mL IVIg group, while both these groups demonstrated a reduction in viral load. This substantiates earlier findings that the functionality of Palivizumab is different from other RSV-specific (monoclonal) antibodies [49].

Our data indicate that the viral load is enhanced in the absence of an apparent increase in the immune response, as judged by pulmonary cellular influx and a limited number of cytokines/chemokines. Potential limitations of our experimental set-up were the assessment of viral and immunological parameters at a single time point (3 dpi) and the absence of histopathological analysis of the lungs. Inclusion of earlier and later time points will elucidate the kinetics of the viral replication and immune response against RSV in the presence or absence of antibodies. This will indicate whether the higher viral load at low antibody concentrations is a result of enhanced infection or reduced viral clearance. In addition, it would be of added value to take along a group that received IVIg depleted of RSV-specific antibodies to prove the effect on viral load and pathology is caused by RSV-specific antibodies. Lastly, mainly the immunological parameters show considerable variation between experiments, which could reflect differences in the ovarian cycle phase [50,51]. Therefore, additional experiments should be performed in male mice.

In conclusion, we have developed a model to study ADE of RSV infection and disease in vivo. We demonstrate that the presence of sub-neutralizing levels of RSV-specific IgG in the lungs can modify the course of infection, resulting in higher viral load and possibly also increased pathology. Additional studies are needed to confirm these findings and unravel the mechanisms underlying these effects. To date, (pre-)clinical tests for RSV vaccines often only demonstrate induction of (neutralizing) antibodies in plasma. In light of the findings presented in the current study, it should be reconsidered whether such a limited assessment of the response to vaccination and infection is appropriate.
REFERENCES


PART V

DISCUSSION
GENERAL DISCUSSION
AND
FUTURE PERSPECTIVES

Why does a minority of infants develop severe disease after RSV infection, whereas the majority remains asymptomatic or only develops mild illness?

The question above is one of the most important unanswered questions in the RSV field. It has occupied RSV researchers since the discovery of the virus and forms the basis of this PhD project. Hospitalization for severe RSV disease peaks in the first 6 months of life [1,2]. During these months, infants mainly rely on both their innate immune system and maternally-derived antibodies for protection. Maternal vaccination is considered as a potential strategy to prevent RSV disease in infants, therefore, it is of pivotal importance to obtain a thorough understanding of the mechanisms by which maternal antibodies can protect against severe disease, both directly and through their interaction with innate immune cells. It has been suggested that high IgA levels in the upper respiratory tract contribute to protection in adults [3]. In infants, a local protective effect of IgA in the upper respiratory tract could be achieved by passive transfer through
However, it is debated whether IgA is the main protective determinant in breastmilk [7]. If antibody levels in the upper respiratory tract are not high enough, RSV infection can disseminate to the lower respiratory tract (Figure 1). Maternal IgG is present in the lower respiratory tract through neonatal Fc receptor (FcRn)-mediated transepithelial transport [8]. High pulmonary antibody levels can neutralize disseminating viral particles and protect against infection. However, low or intermediate antibody levels result in incomplete neutralization, followed by infection of lung epithelial cells and recruitment of immune cells. These events lead to the presence of virus-infected cells, antibodies and immune cells in the lungs, creating the perfect niche for Fc-mediated antibody effector functions. Whether these effector functions are protective or detrimental to the host depends on the degree of activation, which is partly determined by antibody characteristics. However, research on this topic is lacking in the RSV field. In this thesis, we have explored differences in maternal antibody functionality that potentially explain the variation in susceptibility to severe disease.

**FIGURE 1** Interaction between RSV and (local) antibodies in infants

Upon infection, RSV first encounters local antibodies present in the upper respiratory tract (URT). The main antibody subclasses in the mucosa are IgA and IgG that infants obtain through breastfeeding. High mucosal antibody levels will neutralize the virus and protect the individual from infection. Low or intermediate antibody levels will bind the virus in a non-neutralizing manner, resulting in infection of the URT and dissemination to the lower respiratory tract (LRT). Maternal IgG is transferred from the blood to the LRT via the neonatal Fc receptor (FcRn). High pulmonary IgG levels will neutralize the virus and protect from infection. Low or intermediate pulmonary antibody levels do not neutralize the virus, which leads to the infection of airway epithelial cells. RSV-infected cells will secrete cytokines and chemokines, resulting in the recruitment of immune cells to the lungs. The presence of virus-infected cells, antibodies, and immune cells creates a favorable environment for Fc-mediated antibody effector functions. A balanced activation of these effector functions is important, as immune complexes can induce viral clearance leading to mild disease. However, immune complexes can also induce an inappropriate immune response, resulting in inflammation and severe disease.
DISCUSSION

↑ Mucosal antibodies
Neutralization
Protection

↑ Pulmonary antibody levels
Neutralization
Protection

↓ Mucosal antibodies
Infection URT
Dissemination to LRT

↓ Pulmonary antibody levels
No neutralization, infection of epithelium
Recruitment of immune cells
Fc-mediated antibody effector functions

Cytolytic effector functions
Viral clearance
Mild disease

Inappropriate immune response
Inflammation
Severe disease

Primary RSV infection

Mucosal antibody levels
Neutralization
Protection

Pulmonary antibody levels
Neutralization
Protection

Blood
Lumen

Viral clearance
Mild disease

Inflammation
Severe disease

Recruitment of immune cells
Fc-mediated antibody effector functions
THE ROLE OF MATERNAL ANTIBODIES IN RSV INFECTION: EMPHASIS ON FUNCTIONALITY AND IMPLICATIONS IN DISEASE

For nearly all licensed vaccines, antibodies are the presumed correlate of protection, but the underlying mechanisms of protection often remain unknown [9]. Interestingly, in addition to binding and neutralization, antibodies induce Fc-mediated effector functions. However, this potential area of research has been underappreciated in the RSV field. With Chapter 2, we aimed to encourage a paradigm shift from neutralization-based studies toward functional studies examining the precise role of Fc-mediated antibody effector functions in vaccine efficacy and RSV disease. Only a few studies have used FcγR-knockout mice or modified RSV-specific antibodies to elucidate the importance of Fc-mediated effector functions in protection from RSV infection and disease [10-13]. However, data on the effect of antibody functionality in natural RSV infection in humans is lacking and most studies into vaccine and monoclonal antibody efficacy only report antibody (neutralization) titers and disregard Fc-mediated effector functions.

To investigate maternal antibody characteristics that potentially play a role in RSV disease, we employed a case-control study, as described in Chapter 3. This study included RSV patients, RSV-negative infection controls, and uninfected controls below 7 months of age. Using a multiplex immunoassay, we measured plasma IgG levels specific for different RSV antigens. We did not detect a correlation between these antibody levels and RSV hospitalization. These results are in accordance with an earlier study that found no correlation between RSV disease severity and antibody levels, avidity, and neutralization using a subset of these samples [14]. To investigate the involvement of Fc-mediated antibody effector functions, we developed an assay to measure the capacity of RSV-specific maternal antibodies to induce natural killer (NK) cell activity. We used this assay to demonstrate that RSV-specific antibodies, particularly those targeting the G protein, induce NK cell CD107a surface expression and interferon gamma (IFN-γ) production. The capacity of maternal antibodies to induce IFN-γ production was lower in infants with severe respiratory tract infections compared to uninfected controls. Importantly, the capacity to induce NK cell activity was highly variable within the groups indicating that research into additional factors is required.
Fc-mediated antibody effector functions during viral infection do not only contribute to protection, but they can also facilitate infection. Antibodies can target viruses to Fc gamma receptor (FcγR) expressing cells, thereby increasing the number of infected cells. This phenomenon is called antibody-dependent enhancement (ADE) of infection and has been demonstrated in vitro to occur for multiple viruses [15]. Our studies showed that sub-neutralizing concentrations of RSV-specific maternal antibodies induce ADE of infection in vitro and have reduced neutralization capacity on FcγR-carrying cells (Chapter 4). Antibodies from formalin-inactivated (FI-) RSV-immunized cotton rats showed no neutralization, but were potent inducers of ADE of infection in vitro. Although the antibodies from all infants in our cohort had the capacity to cause ADE of infection in vitro, there was no correlation with disease severity. Multiple studies show the presence of RSV in FcγR–expressing cells in vivo [16-18], indicating that ADE of infection should not be ignored as a possible player in RSV disease.

RSV infection of immune cells may have multiple effects, ranging from activation to shutting down cellular effector functions. Any of these effects could lead to the known contribution of immune cells to immunopathology in severe RSV-mediated disease. We were able to demonstrate that RSV-infected NK cells are more prone to produce IFN-γ than uninfected cells, whereas the cytotoxic response is not increased (Chapter 5). Moreover, we show that ADE of NK cell infection augments the effect on IFN-γ production and even inhibits the cytotoxic response. This detrimental combination may contribute to RSV immunopathology in vivo, but more research is needed to support this hypothesis. RSV is not the only virus known to infect NK cells (Chapter 6). The fact that multiple viruses can infect NK cells suggests that this feature can contribute to the establishment of a successful infection. It is essential to expand our knowledge on possible cellular targets and reservoirs of viral infections, as the development of specific therapeutics could benefit from studies into viral infection of different cell types.

Thus far, all the assays we have used in the aforementioned chapters were in vitro cell–based assays. However, to investigate the in vivo relevance of antibody effector functions, we have set up an RSV infection mouse model. Initially, we compared the effect of intratracheal versus intranasal inoculation and demonstrated striking differences with respect to pathological and immunological read–outs (Chapter 7). These differences...
PART V

THE ROLE OF MATERNAL ANTIBODIES IN RSV INFECTION
EMPHASIS ON FUNCTIONALITY AND IMPLICATIONS IN DISEASE

Chapter 2
THE ROLE OF MATERNAL ANTIBODIES IN RSV INFECTION
EMPHASIS ON FUNCTIONALITY AND IMPLICATIONS IN DISEASE

Chapter 3
RSV-specific antibodies induce NK cell activity, which is lower in infants with severe RTI

Chapter 4
In vitro ADE of RSV infection does not explain disease severity in infants

Chapter 5
RSV infects NK cells and affects antiviral effector functions

Chapter 6
Viruses infect NK cells as an immune evasion strategy

Chapter 7
Intranasal inoculation is more appropriate to study RSV disease than intratracheal inoculation

Chapter 8
Sub-neutralizing antibody levels can enhance RSV infection and disease in vivo

FIGURE 2  Summary of the chapters in this thesis
may be explained either by differences in the (initial) site of infection or the
differential use of anesthetics. Based on our findings, intranasal inoculation
appears to be better suited to study RSV-induced immunopathology than
intratracheal inoculation. Using this model to study ADE of RSV infection
and disease \textit{in vivo}, we demonstrate that the presence of sub-neutralizing
levels of passively transferred human IgG in the lungs can modify the
course of infection, resulting in higher viral load and possibly increased
pathology (Chapter 8). Studies are ongoing to further substantiate these
findings and unravel the mechanisms underlying these effects. Figure 2
gives a summary of the different chapters in this thesis.

\textbf{RSV DISEASE IS MULTIFACTORIAL}

There is much debate on the factors that contribute to severe RSV disease,
which is characterized by an inappropriate immune response. Despite
extensive research efforts, the underlying cause of the excessive immune
response remains unknown. In this thesis we point out that antibodies are
important modulators of the immune response (Chapter 2), which can
play both a protective (Chapter 3) and enhancing role in RSV infection
and disease (Chapter 4, 5 and 8). Nevertheless, it is most likely that the
underlying cause for the dysregulated immune response is multifactorial
and driven by additional factors besides antibodies.

Multiple mouse studies have investigated the role of T cells in the
induction or prevention of severe RSV disease. Despite the importance of T
cells in viral clearance, they are paradoxically also suggested to contribute
to tissue injury [19]. Early experiments in mice showed that the transfer
of an RSV-specific T cell line enhanced lung pathology [20]. In addition,
disease after primary infection was diminished by depletion of CD8\(^+\) T cells
[21]. Also in reinfection studies, the pathological role of (memory) CD8\(^+\) T
cells has been indicated [21–23]. However, it is unlikely that (memory) T cell
responses play a major role in severe RSV disease, because hospitalization
most frequently occurs below six months of age when the adaptive immune
response does not yet generate an effective antiviral (memory) response
[24]. It is possible that maternal antibodies are involved in the skewing of the
T cell response, as RSV–antibody immune complexes have been shown to
affect the CD4\(^+\)/CD8\(^+\) T cell balance [25]. Disturbance of this balance could
lead to an exaggerated immune response, resulting in severe disease.
The frequent presence of co-infections may be another underlying cause of the dysregulated immune response during severe RSV disease. Invading pathogens may contribute to an inflammatory milieu that skews the response to an RSV infection. In our cohort, 35% of the hospitalized RSV patients also had other detectable viral pathogens (Chapter 3). Bacterial co-infections were not recorded, but are most probably present in a subset of infants as well. There is increasing interest in the influence of the (respiratory) microbiome on infections and the immune response during an RSV infection, as extensively reviewed by Vissers et al. [26]. A recent study has demonstrated that clinically significant lower respiratory tract infections result from the interplay between host characteristics and (viral and bacterial) respiratory microbiota [27]. In addition, this study suggested that the nasopharyngeal microbiota can serve as a valid proxy for the lower respiratory tract microbiota, enabling the use of less invasive techniques to study the local microbiota. Future studies are needed to unravel the role of the local respiratory (microbial) environment in causing severe RSV disease.

The immunological response after RSV infection may also be affected by age. RSV disease is most frequently seen in the youngest infants, when maternal antibodies are still present. At a certain age, maternal antibodies will be completely gone, but this does not seem to predispose to more disease. Older children may be able to mount a more robust immune response against RSV infection and recover more quickly, even in the absence of any pre-existing immunity. Age may therefore be an important co-factor for RSV disease. Age in this respect is not just “time since birth”, but biological age, indicating the level of maturity of the immune system, the respiratory tract, and probably many other factors.

Taken together, many factors potentially influence disease outcome after an RSV infection. As long as we do not have conclusive knowledge on the underlying causes of severe RSV disease, the multifactorial nature of RSV disease should be taken into consideration when developing model systems and analyzing test results.
THE NEED FOR AN UPDATE OF PRE-CLINICAL MODELS

The development of vaccines against RSV infection is of great importance, especially for developing countries where RSV-related mortality is high and passive immunization with monoclonal antibodies is inaccessible due to high costs. There is an extensive pipeline of vaccines that are being tested in different phases of clinical development (https://www.path.org/resources/rsv-vaccine-and-mab-snapshot/). However, despite the major research efforts to develop effective vaccines, no candidates have gained market-approval so far. The high failure rate of vaccine candidates is not limited to the RSV field, as recent estimates indicate that only 33% of total vaccine candidates that enter phase 1 clinical trials reach market-approval [28]. The success rate is even more dramatic when including all pre-clinically tested vaccine candidates, with only 6% reaching the market [29]. High failure rates are in part due to the use of pre-clinical models that do not accurately represent the in vivo situation. Therefore, improvement of pre-clinical tests is highly needed, especially in the RSV field.

Antibody neutralization tests for RSV: opportunities for improvement

The golden standard for testing the neutralization capacity of RSV-specific antibodies, is the plaque reduction neutralization test (PRNT) [30]. This assay is mostly performed on Vero or HEp2 cells [31]. Both these cell lines do not resemble the natural target cells for RSV, which are airway epithelial cells. The RSV entry receptor on cell lines is heparin sulfate [32], which is not detected on the apical surface of human airway epithelial (HAE) cultures. In these physiologically relevant HAE cultures, CX3CR1 serves as entry receptor for RSV [32]. In addition, HAE cells have been shown to express FcγRs [33], suggesting that sub-neutralizing antibody concentrations can potentially lead to ADE of infection in the human lungs. As Vero and HEp2 cells do not express endogenous FcγRs, assessing antibody neutralization capacity on these cells does not consider the effect of FcγRs, which have been shown to reduce neutralization when expressed on Vero cells (Chapter 4).

The discrepancy in the neutralization capacity between cell lines and primary cells is well illustrated by experiments with RSV G-specific antibodies. Whereas most RSV G-specific antibodies are unable to neutralize RSV infection in immortalized cell lines, these antibodies
are potent inhibitors of infection in primary HAE cultures and in *in vivo* mouse models [32,34–36]. This striking example indicates that a new physiologically relevant model to study antibody neutralization is highly needed. The use of primary HAE cultures in neutralization assays is impractical as these cells demand long differentiation protocols and batches are highly variable due to donor and differentiation differences. Therefore, future research should focus on identifying or constructing cell lines that mimic the human airway epithelium and express all relevant entry receptors for RSV.

In most studies, antibodies originating from the serum are used to measure the neutralizing capacity after infection or vaccination. In accordance with previous studies, we have shown in Chapter 8 that local IgG levels, present in the bronchoalveolar lavage of mice, are approximately 100-fold lower than serum IgG levels. We have also shown in Chapter 4 and 5 that sub-neutralizing concentrations of antibodies may lead to ADE of infection. Maximum enhancement is achieved with 1000-fold serum dilutions, which are concentrations with physiological relevance at the site of infection. Infants that have neutralizing antibody concentrations in their serum could therefore potentially experience ADE of infection in the respiratory tract. Our *in vivo* mouse model, described in Chapter 8, provides indications for enhanced viral load in the bronchoalveolar lavage (BAL) of mice that received a low concentration of IVIg. The concentration in the BAL was 100-fold lower than in the plasma, reaching sub-neutralizing, potentially enhancing concentrations at the site of infection. Consequently, high serum antibody levels are needed to achieve local protection in the respiratory tract. Thus, neutralizing concentrations in the blood are not necessarily a guarantee of neutralizing concentrations at the site of infection. The measurement of local antibody levels in humans is complicated by the lack of non-invasive techniques to sample the lung lumen. However, increased knowledge about antibody transfer rates to the lungs after RSV infection or vaccination could benefit the interpretation of serum neutralization titers. In addition, it should be investigated whether nasal samples can be used as a proxy for lung antibody levels, as this facilitates less invasive sampling techniques. Of note, the expression of FcRn, which facilitates IgG transport to the lungs [8], varies depending on the region in the respiratory tract and may thus lead to differences in local antibody levels [37].
Towards a systems serology approach

Conventional neutralization tests have proven valuable for RSV research in the past. However, RSV-specific neutralization titers correlate poorly with protection for reasons described above. Moreover, additional mechanisms by which antibodies may confer protection against RSV need to be investigated. Mounting evidence suggests that antibody effector functions beyond neutralization can contribute to both protection and disease during RSV infection [10–13]. A balanced activation of different antibody effector functions is essential to prevent excessive inflammation and tissue damage (Chapter 2). It will be of importance to implement RSV-specific assays that determine the functionality of monoclonal and vaccine-induced antibodies.

The NK cell activation assay described in Chapter 3 determines the capacity of antibodies to induce NK cell CD107a surface expression and IFN-γ production. However, this assay is performed with primary human peripheral blood mononuclear cells (PBMCs), complicating standardization and high-throughput applications. This limitation can be overcome by the use of NK cell lines that express FcγRIIIA, which have been developed to study antibody-dependent NK cell responses against tumor cells [38]. In addition, these cell lines were also shown to be successful in studying influenza virus-specific NK cell activation [39]. The development of additional functional assays is of importance for the evaluation and development of future vaccines. This will potentially provide insight in the difference between protective and detrimental antibody profiles in RSV disease.

To comprehensively assess a broad range of antibody characteristics and functions, a systems serology approach can be used. Systems serology systematically surveys humoral immune responses, capturing a broad range of antibody characteristics and functions [40,41]. It consists of multiple high-throughput in vitro assays, followed by computational methods to examine the multiple layers of information. Antibody characteristics that are assessed include subclass, isotype, glycosylation, epitope specificity, and affinity (for the antigen as well as the Fc receptor). The functional assays in these approaches often measure the extent of neutralization, NK cell activation (IFN-γ and MIP-1β secretion, and CD107a surface expression), antibody-dependent cellular phagocytosis (ADCP) by both monocytes and neutrophils, complement deposition, and cytokine release by various
immune cells [40,41]. However, numerous additional factors may be taken into account, as up to 70 humoral features have been measured within one study [42]. The incorporation of all these factors together allows for the identification of features or patterns within the antibody response that distinguish protective from non-protective immunity.

A systems serology approach has been used to identify antibody features that contribute to protection against Ebola virus infection [43]. In addition, this approach was used to distinguish between patients with latent tuberculosis infection and active tuberculosis disease [42]. Both studies demonstrate that the integration of multiple characteristics and functionalities is needed, as no single assay was able to predict protection. In addition to the unbiased evaluation of multiple factors at once, the requirement of only low amounts of sample is another benefit of a systems serology approach. This allows profound analysis of precious samples with only limited volume, such as those from infants. Of course, systems serology leads to the acquisition of enormous amounts of data, indicating the need for researchers that are skilled in bio-informatics and modelling. The demand for these skills will only grow as the generation and availability of big data will rapidly increase in the future.

Systems serology is still in its early developmental stages. New insights in antibody functionality and improved analytical methods will contribute to the improvement of the technique. The broader the range of antibody features that is included, the more comprehensive our understanding of desirable antibody profiles will be. This will help to guide rational design of future vaccine candidates. An important antibody feature that has not been included in systems serology approaches so far is the binding capacity to FcRn. FcRn extends the half-life of serum antibodies [44] and is essential in the transport of antibodies across the lung epithelium [8]. Determining the FcRn binding capacity may therefore provide important clues about local antibody levels in the lungs. Another antibody feature that should be included is specificity at an epitope level. A method recently developed by Xu et al. has revolutionized high throughput serological profiling, allowing the identification of the complete antibody response at epitope level [45]. This approach has recently been used to demonstrate that preterm and term infants receive a comparable repertoire of maternal RSV-specific antibodies, although preterm infants receive a lower amount of these maternal antibodies [46]. Incorporating this “Virscan” method in a
systems serology approach could provide essential information about the difference between a beneficial and detrimental antibody specificity profile.

**Tailor-made animal models for the investigation of antibody effector functions**

Much of the information on the RSV immune response comes from animal models, due to the difficulty to study the immune response against RSV in infants because of both ethical and practical considerations. The limited information that is available originates from the most severe cases that require hospitalization, which may not be representative for the mild disease seen in most infants. Moreover, by the time of hospitalization, the trigger for severe disease may already be gone. In addition, investigating the local immune response is challenging, as sampling the response in the respiratory tract is invasive and challenging in infants that suffer from RSV disease. This makes it necessary to use animal models to investigate the immune response against RSV.

The most widely used animal models are inbred mouse strains, because of the availability of immunological reagents as well as the possibility to genetically modify these animals. However, most inbred mouse strains, including the widely used BALB/c and C57Bl/6 mice, are only semi-permissive hosts for RSV infection. Mice need a high viral inoculum to detect any lower respiratory tract disease symptoms and general measures of illness such as weight loss [47,48]. In addition, the innate and adaptive immune responses after RSV infection differ greatly between humans and mice. For example, mice show a limited recruitment of neutrophils to the lungs, whereas neutrophils are the most abundant cell type during human RSV infection [47,49]. Another important discrepancy between natural infection in humans and experimental infection in mice is the presence of RSV-specific antibodies. RSV infections in humans practically always occur in the presence of (maternal) RSV-specific antibodies, whereas most mouse studies are performed in RSV-naive mice. The presence of antibodies can have a major influence on RSV infection, as we have shown in Chapter 8, and should therefore not be ignored when using RSV-naive mouse models.

There are considerable differences in FcγR expression and affinity between mice and humans. To assess antibody functionality in vivo, the use of mice with a humanized immune system (HIS) or mice with humanized
**FcγRs may ease the translation between mouse studies and the response in humans.** HIS mice are generated by the introduction of human HLA class II genes, various human cytokines, and human B cell activation factor into non-obese diabetic/severe combined immunodeficiency gamma mice, followed by engraftment of human hematopoietic stem cells [50]. Sharma et al. demonstrated that HIS mice mimic RSV disease in humans and induce human anti-RSV antibody responses [51]. However, further development of this mouse model is needed, as the CD8+ T cell response in these mice is nonfunctional. In addition, use of HIS mice in functional antibody studies neglects the biological functions of human FcγRs at sites other than immune cells. Expression of FcγRs on nasal epithelial cells has recently been demonstrated [33] and may be of importance in respiratory infections in the presence of pre-existing (maternal) antibodies. Therefore, the use of transgenic mice expressing human FcγRs may be superior in translational research into antibody effector functions [52]. However, the currently available transgenic mouse models do not express all human FcγRs [53], indicating that also for this mouse model further development is needed. To our knowledge, no attempts to investigate RSV antibody effector functions in transgenic FcγR mice have been published.

Although most widely used, mice are not the only available animal model in the RSV research field. Cotton rats have been instrumental in the testing of vaccines and monoclonal antibodies [54]. They are approximately 100-fold more permissive to RSV infection than BALB/c mice and show vaccine-enhanced disease after immunization with FI-RSV [55]. However, cotton rats do not develop clinical signs of disease after natural infection and species-specific (immunological) reagents and molecular tools are lacking, impeding mechanistic studies of RSV immunity in this animal model. The downside of the semi-permissive nature of human RSV infection in animal models can be solved by the use of related non-human pneumoviruses in their native host. Although studies with bovine RSV (BRSV) and pneumonia virus of mice (PVM) have increased our understanding of viral pathogenesis (as reviewed in [56]), the differences between the species-specific immune responses limit the relevance to RSV disease in humans. Additionally, transfer of maternal antibodies to calves exclusively takes place via the colostrum [57], indicating that cattle are an unsuitable model to study the role of (vaccine-induced) maternal antibodies.

Besides the necessity to use tailor-made animal models for specific
research questions, also the reporting in these experimental models urgently needs improvement. Most animal studies on vaccine or antibody responses only report on inhibition of viral load, whereas an excessive immune response has also been suggested to contribute to severe disease. Measures of immunopathology such as pulmonary cellular influx, lung damage, local cytokine and chemokine secretion, or immune cell activation should be included. In our studies, we have shown that viral load is lower in the presence of high dose IVIg but both pulmonary cellular influx and cytokine responses remain high, indicating that viral load and immunopathology are not always associated (Chapter 8). These findings are in accordance with studies in cotton rats, showing decreased viral load but no decrease in inflammation markers in animals that received Palivizumab [58,59].

As described above, animal models of RSV do not mimic all aspects of human disease, which may hamper investigation of the RSV immune response. Investigation of hospitalized infants allows the observation of natural disease, but does not provide insight in the immune status before infection and its correlation with susceptibility. Due to the mild symptoms of natural RSV infection in healthy adults, experimental human challenge models can be used to offer important insights in pre-existing immunity and the kinetics of the (early) immune response. Experimental RSV infection of healthy adults has elucidated the protective effect of mucosal IgA responses and indicated that a defective IgA B cell memory explains the recurrent RSV infections seen throughout life [3,60]. To study RSV-specific (protective) immune responses during natural infection, the human challenge model is the ultimate model. However, as challenge models only include well-informed adult volunteers, primary infections in presence of maternal antibodies, but in absence of a cellular memory response, cannot be mimicked. In addition, depletion and knockout studies are instrumental to unravel the contribution of specific factors to protection and disease. Therefore, animal models are still irreplaceable in this respect.
DEVELOPMENT OF FUTURE VACCINES

Thus far, no RSV vaccine candidates have proven successful enough for market-approval. As described above, preclinical testing needs improvement to recognize promising vaccine candidates more efficiently. In addition, research efforts into potential correlates of protection and rational vaccine design are highly necessary.

The lack of accurate correlates of protection hampers the development of new vaccines, as efficacy now has to be demonstrated in expensive large-scale clinical trials. It is important to realize that natural RSV infection only temporarily protects against re-infection by the induction of a short-lived protective mucosal IgA response [3,60]. Natural infection apparently does not induce a long-lived protective immune response, indicating that we may need to look at clinical trials with various vaccine concepts to find potential correlates of protection. Most importantly, this demonstrates that future vaccines need to induce a superior immune response compared to natural RSV infection. Increasing knowledge on the distinction between protective and detrimental immune responses will help in the rational design of vaccines.

Lessons for vaccine design

The majority of vaccine candidates currently in clinical trials are designed to induce systemic (neutralizing) antibodies, mostly against the RSV F protein. As described earlier in this chapter, this presents multiple problems. First, vaccine-induced antibodies need to be tested for more functional characteristics than just neutralization capacity. A more polyfunctional antibody response may contribute to protection against infection as has been shown in HIV vaccine trials [61–63]. Second, systemic antibody characteristics are not necessarily representative for local lung antibodies. Only certain antibody isotypes and subclasses are readily transferred across the lung epithelium, and lung antibody levels are approximately 100-fold lower than serum antibody levels (Chapter 8). In addition, RSV-specific locally-derived (adenoid) antibodies have higher binding affinity and neutralizing capacity compared to antibodies from the blood [64]. Whether there are additional functional differences between serum antibodies and local antibodies in the lungs remains to be determined. An efficacious vaccine should induce a preferably long-lasting, local antibody response in the lungs and/or induce...
antibodies that readily bind FcRn and are efficiently transported from the serum. Third, the recent failures of two (post-) F–specific vaccine candidates tested in elderly, show that a broader specificity of the immune response may be needed to confer protection against RSV-mediated disease [65] (press release Novavax, September 15, 2016). The RSV G protein has long been ignored as a potential vaccine antigen, but is now increasingly recognized as a critical target in vaccine development [66]. The G protein was shown to be a target for broadly neutralizing antibodies [36,67] and G–specific antibodies are the major contributors to NK cell activation (Chapter 3) [68]. Besides its involvement in the attachment of viral particles to target cells, the G protein is known to modulate the immune response through interaction with CX3CR1 [69]. The induction of G–specific antibodies may thus be important to reduce immunomodulation by the RSV G protein. With currently as many as 21 different vaccine candidates in clinical trials (https://www.path.org/resources/rsv-vaccine-and-mab-snapshot/), the results of these studies will provide us with additional clues for the design of an effective RSV vaccine.

Infants as target population: The rationale for maternal vaccination
Most of the current RSV vaccine candidates have a pediatric target indication. However, vaccination at this young age has several downsides. The generation of T and B cell memory in infants is poor due to impaired antibody affinity maturation and less efficient antigen presentation, resulting in inappropriate adaptive immunity after early vaccination [24]. Thus, multiple vaccinations will be necessary to induce an adequate immune response. In addition, the presence of maternal antibodies may inhibit the vaccine response at an early age, as they may have an immunosuppressive effect on the (humoral) immune response against RSV [70–73]. An alternative strategy to protect infants at early age is the vaccination of pregnant women, which will boost the mother’s antibody response as illustrated in Figure 3. This will lead to an increased transplacental transfer of specific antibodies to the blood of the unborn child. Increased serum antibody levels result in increased lung antibody levels, which can provide passive immunity during the first months of life. A study investigating the decline of naturally acquired RSV–specific maternal antibodies suggested that maternal vaccination could provide protection in infants for at least 3 – 6 months [74].
Vaccination during pregnancy will boost the maternal immune response leading to increased levels of vaccine-induced immune cells and antibodies in the maternal blood. The neonatal Fc receptor (FcRn) facilitates the transfer of IgG from maternal blood to fetal blood. Immune cells are not able to cross the placental barrier, therefore, the unborn child will only receive humoral maternal immunity.
To date, two maternal anti-RSV vaccines have been tested in clinical trials. The first vaccine candidate was an adjuvanted purified F protein, which was reported to be safe and well tolerated [75]. However, a more immunogenic vaccine was desired as the functional neutralizing antibody response was disappointing. The other vaccine candidate is an alum-adjuvanted F protein nanoparticle vaccine [76]. This recombinant F protein has a prefusion-‘like’ conformation (Smith 2012) ([https://novavax.com/download/files/presentations/NOVAVAX-WVC_RSV_2018APR3-FINAL.pdf](https://novavax.com/download/files/presentations/NOVAVAX-WVC_RSV_2018APR3-FINAL.pdf)). The only outcome measures for the phase 2 clinical trial were anti-F IgG antibody titers, Palivizumab-competitive antibody levels, and microneutralization titers [77]. No additional antibody characteristics or functions were reported. The first results of the phase 3 clinical trial show that the primary efficacy endpoint (reduction of medically-significant RSV lower respiratory tract infection) was not met ([https://novavax.com/presentation.show](https://novavax.com/presentation.show)). However, the vaccine did demonstrate a significant reduction in hospitalization and severe hypoxemia. An interesting finding that warrants further investigation is the geographic imbalance in efficacy: whereas vaccine efficacy appeared to reach 57% in South Africa, it was −32.7% in the U.S., suggesting that more infants acquired medically-significant RSV disease in the vaccinated group compared to the placebo controls. Therefore, besides not meeting the primary endpoint, the induction of solely pre-F-‘like’ antibodies could potentially prime for vaccine-enhanced disease. A possible explanation for these geographical differences could be the frequency or duration of breastfeeding. It must be noted that the wide confidence intervals of the vaccine efficacy in this study complicate the interpretation of the results. Further analysis will have to provide more definitive conclusions on this trial, but these data prompt a thorough examination of the underlying causes of the increase in cases in the vaccine group.

Besides transplacental IgG transfer during pregnancy, infants can also acquire maternal IgA, IgG, and IgM postnatally through breastfeeding. Transfer of maternal antibodies through breastfeeding is known to increase protection against respiratory tract infections [78,79]. For RSV infection, decreased hospitalization rates are demonstrated in breastfed infants [4,5], whereas another study indicates reduced disease severity in breastfed infants, but no effect on hospitalization rates [6]. In addition, pre-F protein–specific IgG levels, but not IgA levels, were lower in breastmilk fed
to RSV-infected infants compared to healthy controls [7]. These studies indicate that maternal vaccination could also improve the protective effect of breastfeeding. Novavax’ recent clinical trial may provide insight in the protective role of vaccine-induced antibodies from breastmilk.

The goal of maternal vaccination to induce high maternal antibody levels in infants also has a downside: The higher the maternal antibody levels, the longer it takes until the child’s own humoral immune response is effective due to interference of the maternal antibodies [57]. Maternal vaccination might therefore only delay the age at which infants are vulnerable to infection, whereas the length of the vulnerable period will stay the same. However, delay of disease may already provide substantial clinical benefit, as older infants may cope better with RSV infection [1].

**The perfect RSV vaccine to prevent disease in infants**

There is no such thing as ‘the’ perfect RSV vaccine. The ideal formulation of the vaccine greatly depends on the target population and desired goal. Completely preventing RSV infection may not be feasible; therefore, prevention of severe RSV disease should be the major goal of RSV vaccine development. Currently, maternal vaccines are not specifically designed for the use in pregnant women. However, a design specifically catered for maternal vaccination should be considered. Due to the FcRn-restricted transplacental transfer, IgG is the main isotype that is transported to the unborn child [80]. Of all IgG subclasses, IgG1 is transferred most efficiently, whereas IgG2 is not [81]. A maternal vaccine should therefore induce a robust IgG1 response. This has implications for vaccine design, as the response to protein antigens induces class switching to IgG1 or IgG3, whereas polysaccharide antigens induce class switching to IgG2 [82]. Potent FcRn binding also facilitates recycling of the antibodies in the infant, resulting in a longer half-life.

Concerning specificity, a maternal vaccine should primarily consist of the RSV surface proteins (pre-) F, G, SH, and N. Although the RSV N protein was initially recognized to be an internal protein, its expression on the surface of RSV virions and infected cells has recently been demonstrated [83] and high anti-N (maternal) antibody levels have been demonstrated in plasma (Chapter 3). Antibodies against the pre-fusion conformation of the F protein were shown to be the most potent neutralizers [84], therefore, the pre-F protein is indispensable in the vaccine design. Antibodies to the
RSV G protein also induce some neutralizing activity, and additionally block the immunomodulatory activity of the secreted G protein [85]. Instead of using the native G protein in the vaccine, inclusion of an adapted form of the G protein may be beneficial to prevent its contribution to disease pathogenesis [86]. A recent study demonstrated that only a small domain of the G protein is responsible for the majority of anti-RSV G antibodies [46], indicating that only part of the G protein may be needed to elicit an efficient antibody response.

The antibody response induced by maternal vaccination should be tested for FcRn binding and transport, virus neutralization, cytolytic functionality, and complement fixation capacity before use in the target population. Complement fixation should be prevented, as antibody–complement deposition has been shown in RSV vaccine–enhanced disease [87]. T cells are not transplacentally transferred; therefore, an effector T cell response is not the major goal in maternal vaccination. However, a T (helper) cell response is needed to optimize the humoral response and is thus, indirectly, important during maternal vaccination.

The most ideal vaccine platform for maternal vaccination may be the use of nanoparticles or viral–like particles (VLPs), as these have been shown to elicit high specific antibody responses against human papilloma virus in the target population [88]. Recently, an RSV pre–F–specific nanoparticle vaccine was developed, which demonstrated superior (neutralizing) responses in animal models compared to the use of soluble single antigens [89]. Whereas this nanoparticle vaccine is composed of pre–F proteins only, nanoparticle vaccines and VLPs can be generated in such a way that they contain multiple RSV (surface) proteins, thus eliciting a broadly specific antibody response. While the current maternal vaccine programs recommend non–adjuvanted vaccines, the use of adjuvants may be useful to modulate antibody characteristics that regulate transport, half–life and functionality. Therefore, future research should also focus on the effect of adjuvants on the antibody characteristics described above.
The findings described in this thesis, together with an extensive review of the current literature, lead to certain recommendations for the RSV field. The following points should be considered in future RSV research initiatives:

1. Current *in vitro* neutralization tests should be improved to reflect the physiological situation in the lungs.

2. To predict local protection, assays to measure lung antibody levels are needed, in addition to an increased understanding of the factors that determine the ratio between serum and lung antibody levels.

3. Antibody functionality besides neutralization should be explored as potential (co-)correlate of protection. Systems serology captures a wide array of antibody characteristics and effector functions and may be useful in identifying antibody features that contribute to protection.

4. Although antibodies are known to modulate the immune response, other factors are likely to be involved in RSV disease and should be investigated as potential correlates of disease.

5. Human challenge studies and vaccine evaluations in preclinical animal models need to report on immune readouts, as an excessive immune response is an important factor underlying severe RSV disease. Early elimination of vaccine candidates that are not effective in decreasing immunopathology will decrease the failure rate in clinical trials.

6. The ideal formulation of the vaccine greatly depends on the target population and desired goal. Designs of future vaccines should be specifically catered for their target population. Maternal RSV vaccines should induce a protective antibody response against the RSV surface proteins that is efficiently transferred to the unborn child.
CONCLUDING REMARKS

In conclusion, the research described in this thesis has provided novel insights in the role of antibody effector functions during RSV infection. We have developed several assays to investigate RSV-specific Fc-mediated antibody effector functions, some of which are thought to be protective in nature, whereas others potentially contribute to disease. A better understanding of the broad range of effector mechanisms that are induced by RSV-specific antibodies will greatly contribute to the much-needed development and testing of next generation vaccines against this virus. Future research should focus on integrating a broad range of antibody features, using a systems serology approach, to distinguish between protective and detrimental antibody responses.
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DISCUSSION


73. Trento, A., et al., The Complexity of Antibody Responses Elicited against the Respiratory Syncytial Virus Glycoproteins in Hospitalized Children Younger than 2 Years. Front Microbiol,


ADDENDUM
Virussen zijn interessante ziekteverwekkers. Ze bestaan slechts uit genetisch materiaal (zoals DNA of RNA) dat goed is ingepakt in een beschermende eiwitmantel. Toch kunnen virussen ontzettend veel schade aanrichten in een individu of zelfs in hele populaties, zoals in het verleden is gebeurd met de uitbraak van Spaanse griep of SARS, en recent met de uitbraak van Ebola. Bij een virusinfectie dringen virussen de cellen van de gastheer binnen om daar te vermenigvuldigen. Hierbij ontstaat vaak schade aan deze cellen waardoor weefsel en organen minder goed gaan functioneren. Dit kan leiden tot ziekte of zelfs tot overlijden van de geïnfecteerde persoon. Hoewel er heel wat vaccins op de markt zijn tegen virusinfecties (denk aan mazelen, bof of polio), zijn er verschillende virussen waar we nog vrij weinig tegen kunnen doen, terwijl ze wel ernstige ziektes kunnen veroorzaken. Over één van die virussen gaat dit proefschrift: het respiratoir syncytieel virus.

Respiratoir syncytieel virus, ook wel RSV of RS-virus genoemd, is een virus dat vooral in het winterseizoen voor veel last zorgt. In gezonde kinderen of volwassenen veroorzaakt het virus slechts een verkoudheid met milde symptomen, maar in jonge kinderen (onder de zes maanden) kan een infectie leiden tot ernstige luchtweginfecties en zelfs longontsteking. Ondanks dat er al lange tijd onderzoek gedaan wordt naar RSV, is er nog steeds geen behandeling of vaccin beschikbaar. In Nederland worden elke winter duizenden kinderen opgenomen in het ziekenhuis met een
ernstige RSV infectie, maar door de goede gezondheidszorg komt sterfte hier niet veel voor. Helaas is dat wereldwijd gezien anders en sterven ruim 100.000 kinderen per jaar aan de gevolgen van een RSV infectie. Vandaar dat het belangrijk is om onderzoek naar dit virus te doen en testen te ontwikkelen om de werkzaamheid van toekomstige vaccins beter te kunnen beoordelen.

Van alle jonge kinderen die geïnfecteerd raken met RSV in hun eerste levensjaar, wordt maar een klein deel ernstig ziek. We weten nog steeds niet precies waarom deze jonge kinderen zo ziek worden en dat maakt het ontwikkelen van een vaccin tegen RSV extra moeilijk. Er zijn aanwijzingen dat een verkeerde activatie van het immuunsysteem een grote rol speelt in het ontstaan van een ernstige RSV infectie. Essentieel in het immuunsysteem van een pasgeborene zijn de zogenaamde maternale antistoffen die tijdens de zwangerschap via de placenta worden overgedragen van moeder naar het (dan nog ongeboren) kind.

Antistoffen zijn Y-vormige eiwitten die door het hele lichaam aanwezig zijn. Vooral in het bloed vinden we veel van deze antistoffen, maar bijvoorbeeld ook in de longen; de plek waar RSV infecties plaatsvinden. Antistoffen worden gemaakt door het immuunsysteem met als doel om aan lichaamsvreemde stoffen, zoals virussen of bacteriën, te binden (zoals weergegeven in Figuur 1). De binding van antistoffen aan ziekteverwekkers kan vervolgens op verschillende manieren bescherming bieden. Een van de meest bekende functies is neutralisatie: hierbij wordt het virus volledig omgeven door antistoffen, waardoor het geen cellen meer kan infecteren. Er bestaan echter nog veel meer, vaak minder bekende, functies van antistoffen. Welke functie door de antistoffen wordt uitgevoerd, hangt onder andere af van het aantal antistoffen dat bindt, de kenmerken van die antistoffen (welk type antistof en hoe goed ze binden) en de staat van het immuunsysteem. Het onderzoek dat in dit proefschrift wordt beschreven, focust op de rol van verschillende functies van maternale antistoffen tijdens een RSV infectie. We kijken of bepaalde antistoffuncties bijdragen aan bescherming of misschien juist bijdragen aan een ernstiger ziekteverloop.

Na een korte introductie in hoofdstuk 1, geven we in hoofdstuk 2 een overzicht van de verschillende antistoffuncties waarvan bekend is dat
FIGUUR 1  Schematische weergave van de vorming van RSV-antistof complexen.
ze een rol kunnen spelen in virale infecties. In dit hoofdstuk beschrijven we het onderzoek dat wereldwijd is uitgevoerd op het gebied van antistofffuncties. Op het gebied van RSV blijkt dat er nog vrij weinig bekend is over antistofffuncties, en is er dus nog veel kennis te behalen. Met het onderzoek dat in de rest van dit proefschrift beschreven wordt, leveren wij een bijdrage aan het vergroten van deze kennis. De volgende hoofdstukken van dit proefschrift beschrijven het onderzoek dat we hebben uitgevoerd om te kijken naar verschillende antistofffuncties. In hoofdstuk 3 laten we zien dat RSV-antistoffen bepaalde immuuncellen, genaamd natural killer (NK) cellen, kunnen activeren. Deze NK cellen zijn onderdeel van ons aangeboren immuunsysteem en belangrijk voor het opruimen van virusinfecties. Wanneer we antistoffen van kinderen met een ernstige virale infectie vergelijken met de antistoffen van gezonde kinderen, dan zijn de antistoffen van zieke kinderen minder goed in het activeren van NK cellen. Dit is een aanwijzing dat NK cel activatie door antistoffen belangrijk is voor de bescherming tegen ernstige infecties. Behalve hun bijdrage aan bescherming, kunnen antistoffen in sommige gevallen ook bijdragen aan ziekte. Er werd altijd gedacht dat RSV vooral longcellen infecteert, maar in hoofdstuk 4 laten we zien dat de aanwezigheid van antistoffen soms ook kan leiden tot een verhoogd aantal geïnfecteerde immuuncellen. Dit vindt met name plaats wanneer er wel antistoffen aanwezig zijn, maar te weinig om het virus volledig te neutraliseren. Om te voorkomen dat antistoffen de infectie bevorderen in plaats van bestrijden, is het dus belangrijk dat toekomstige vaccins zorgen voor de juiste antistoffen en dat de concentratie van deze antistoffen hoog genoeg blijft om te beschermen tegen ernstige ziekte.

Na de ontdekking dat RSV immuuncellen kan infecteren, en dat een lage concentratie antistoffen kan bijdragen aan dit proces, hebben we voor hoofdstuk 5 uitgezocht wat het effect van deze infectie is op het functioneren van immuuncellen. We hebben in het laboratorium NK cellen geïnfecteerd met RSV en zagen dat deze cellen wel een ontstekingsreactie kunnen veroorzaken, maar minder goed waren in het opruimen van de infectie in vergelijking met niet-geïnfecteerde NK cellen. Het is dus goed mogelijk dat deze combinatie van niet goed opruimen én een verhoogde ontstekingsreactie bijdraagt aan het ontstaan van een longontsteking. RSV is niet het enige virus dat NK cellen kan infecteren. In hoofdstuk 6
belichten we 13 andere virussen waarvan bekend is dat ze NK cellen infecteren en beschrijven we hoe deze virussen het functioneren van NK cellen aantasten.

Voor het laatste deel van dit proefschrift hebben we gekeken of onze eerdere bevindingen in celkweekmodellen ook in een levend organism platsvinden. Omdat het niet goed mogelijk is om het effect van antistoffen op een RSV infectie in mensen te testen, hebben we een diermodel ontwikkeld. We maken hiervoor gebruik van muizen omdat de ontstekingsreactie goed bestudeerd kan worden en ze een veelgebruikt infectiemodel zijn voor RSV onderzoek. In hoofdstuk 7 laten we zien dat muizen het beste via druppels in de neus met RSV geïnfecteerd kunnen worden. Deze toedieningsroute leidt tot hogere infectiewaarden, meer symptomen en een hogere immuunrespons in vergelijking met een toediening direct in de longen. Dit heeft belangrijke implicaties voor alle laboratoria die het muismodel gebruiken voor onderzoek naar RSV infecties. In hoofdstuk 8 beschrijven we hoe we dit muismodel hebben ingezet om het effect van antistoffen op het verloop van een RSV infectie te bepalen. Muizen kregen verschillende concentraties antistoffen toegediend, waarna ze geïnfecteerd werden met RSV. Zoals verwacht lieten de muizen die een hoge concentratie antistoffen hadden gekregen lagere infectiewaarden zien dan de dieren zonder antistoffen. Maar in muizen met een lage concentratie antistoffen zagen we juist hogere infectiewaarden, in vergelijking met muizen zonder antistoffen. Dit resultaat was vergelijkbaar met wat we in hoofdstuk 4 al in celkweekmodellen hadden gevonden. Het lijkt er dus op dat het belangrijk is om óf een hoge concentratie antistoffen te hebben, óf helemaal geen antistoffen, omdat een lage concentratie antistoffen mogelijk juist bijdraagt aan het ontstaan van een ernstig ziektebeloop.

Concluderend geeft ons onderzoek inzicht in de rol van antistoffuncties tijdens RSV infecties. We hebben verschillende methoden ontwikkeld om deze functies te onderzoeken en laten zien dat sommige antistoffuncties waarschijnlijk beschermend werken, terwijl anderen misschien juist bijdragen aan de ernst van ziekte. Een beter begrip van alle verschillende antistoffuncties die een rol spelen tijdens RSV infectie, zal bijdragen aan de ontwikkeling en het testen en beoordelen van nieuwe vaccins tegen dit virus.
CURRICULUM VITAE

Elisabeth Anna (Liz) van Erp was born on March 29th 1990 in Oss, the Netherlands. She grew up in Heesch and finished her secondary school (VWO) at the Titus Brandsma Lyceum in 2008. Subsequently, she took a gap year to travel around New Zealand, before starting her bachelor studies in Biomedical Sciences at Utrecht University in 2009. During these studies, she also completed a minor in Journalism at the Utrecht School of Journalism. She was an active member of the student rugby club RUS, and played for the Dutch women’s national rugby team next to her studies. After obtaining her degree Bachelor of Science in 2013, she enrolled in the master program Infection and Immunity at Utrecht University.

During her master studies, she performed her first research internship at the Department of Pathology of the University Medical Centre in Utrecht (UMCU) under the supervision of Dr. Niels Bovenschen, where she developed a sensor for the detection of Granzyme M activity. For her second research internship, she went to Washington University Medical School in St. Louis (USA). Here, she worked in the lab of Prof. Dr. Curiel where she contributed to the development of an oncolytic (tumor-killing) virus. This internship convinced her to pursue a PhD after obtaining her degree Master of Science *cum laude* in 2015.

In May 2015, she started as a PhD candidate at the Centre for Immunology of Infectious Diseases and Vaccines of the National Institute for Public Health and the Environment (RIVM) in collaboration with the department of Pediatric Infectious Diseases at the Radboud University Medical Centre. Her supervisors were Dr. Oliver Wicht, Dr. Puck van Kasteren, Dr. Gerben Ferwerda, Prof. Dr. Ronald de Groot, and Prof. Dr. Debbie van Baarle. The research of her PhD project focused on the effector functions of RSV-specific maternal antibodies and their implications in RSV disease. During her PhD, she participated in an educational research program for primary schools to promote vaccine education. The results obtained during her PhD are described in this thesis and published in peer-reviewed scientific journals.
PHOTO  Portrait of Liz van Erp

Photographer: Sander Koning, Location: RIVM, June 2018
LIST OF PUBLICATIONS

IN THIS THESIS

E.A. van Erp, W. Luytjes, G. Ferwerda, and P.B. van Kasteren
Pathogenesis of respiratory syncytial virus infection in BALB/c mice differs between intratracheal and intranasal inoculation
Viruses · 2019
https://doi.org/10.3390/v11060508

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*Blood* • 2015  
https://doi.org/10.1182/blood-2015-07-657841

*Molecular Therapeutics – Oncolytics* • 2015  
https://doi.org/10.1038/mto.2015.1
# PHD PORTFOLIO

**LIZ VAN ERP**

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## TRAINING ACTIVITIES

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</tr>
<tr>
<td>Proneri PhD Retreat – Utrecht (2017*)</td>
<td>2017</td>
<td>0.75</td>
</tr>
<tr>
<td>RSV Vaccines for the world – Malaga, Spain (2017*)</td>
<td>2017</td>
<td>1.25</td>
</tr>
<tr>
<td>ASV Annual meeting – College Park, USA (2018*)</td>
<td>2018</td>
<td>1.5</td>
</tr>
<tr>
<td>European Congres of Immunology – Amsterdam (2018*)</td>
<td>2018</td>
<td>1.25</td>
</tr>
<tr>
<td>European Congres of Virology – Rotterdam (2019*)</td>
<td>2019</td>
<td>1.25</td>
</tr>
</tbody>
</table>

| Other | | |
| Journalclub IIV (RIVM) | 2015–2019 | 1.0 |
| Peer review of scientific paper | 2019 | 0.1 |
# TEACHING ACTIVITIES

<table>
<thead>
<tr>
<th>Lecturing</th>
<th>Year(s)</th>
<th>ECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onderzoeker in de klas</td>
<td>2017-2019</td>
<td>0.3</td>
</tr>
<tr>
<td>Primary school education: “Immunology &amp; vaccination”</td>
<td>2018</td>
<td>0.1</td>
</tr>
<tr>
<td>Lecture series “From DNA – protein” (Life Science Leiden)</td>
<td>2017</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Supervision of internships**

| Student supervision                                                        | 2016-2018   | 6    |

**Other**

| Career- and internship market Mebiose (Utrecht)                            | 2016-2019   | 1.5  |
| Incl. Workshop: “Research at the RIVM”                                     |             |      |
| Tour + lectures for honours students UU                                    | 2019        | 0.5  |

**TOTAL** 45.3

* Oral presentation
* Poster presentation
DANKWOORD

Tot slot is het tijd voor het dankwoord: Het enige deel uit een proefschrift dat niet gecheckt en verbeterd wordt door je begeleiders en waar ik dus helemaal los kan gaan! In mijn eentje had ik dit hele PhD traject nooit kunnen volbrengen en daarom wil ik graag iedereen bedanken die de afgelopen vier jaar een steentje heeft bijgedragen aan dit grote geheel.

Beste Oliver en Puck, voor jullie een gedeelde eerste plaats. Oliver, jij hebt ervoor gezorgd dat ik voor dit PhD project gekozen heb; we hadden tijdens het sollicitatiegesprek al zoveel ideeën en nieuwe onderzoeks-vragen bedacht, dat ik mijn idee om in de VS te promoveren liet varen. Onze samenwerking bleef ook tijdens mijn PhD project erg soepel en we hebben lange dagen in het lab gemaakt, o.a. om “tizzle-idel-fizzles” te doen. Helaas was het voor jou na 1,5 jaar tijd om een volgende carrière stap te maken en moest je het maternale antilichamen project en je eerste PhD student achterlaten. Ik ben blij dat we nog steeds contact hebben en dat ik er een vakantieadresje in Londen bij heb! Toen Oliver wegging, was het aan jou, Puck, om mijn project verder te begeleiden. Het was geen makkelijke start, aangezien het (maternale) antilichamen onderzoeksveld nieuw voor je was en je ook nog niet zo lang aan RSV werkte. Maar uiteindelijk zijn we echt een super goed team geworden en hebben we de laatste jaren heel veel voor elkaar gekregen samen. We hebben mooie tripjes gemaakt, veel mensen leren kennen en ik heb ontzettend veel van je geleerd. Onwijs bedankt voor alles!

Beste Gerben, jouw ideeën raakten nooit uitgeput. We zagen elkaar niet vaak, maar áls we een meeting hadden, kwam ik daar met een hoofd vol inspiratie weer uit. Ik ben heel blij dat ik in NK cellen ben blijven geloven, maar om eerlijk te zijn ben ik ervan overtuigd dat dat niet de enige belangrijke cellen zijn. Jouw voorliefde voor monocyten en macrofagen kan dus best nog wel eens tot interessante bevindingen leiden: Ik ben heel benieuwd!
Natuurlijk kun je niet promoveren zonder promotor(en). Ronald en Debbie, dankzij jullie is het eind van mijn PhD een stuk soepeler verlopen dan ik had durven hopen en zijn mijn “ambitieuze plannen” werkelijkheid geworden. Bedankt daarvoor!

Leden van de manuscriptcommissie, prof. dr. Annemiek van Spriel, prof. dr. Louis Bont en prof. dr. Frank van Kuppeveld, hartelijk bedankt voor de tijd die jullie hebben genomen voor het beoordelen van mijn proefschrift. Ook de rest van de promotiecommissie wil ik bedanken voor hun tijd en aanwezigheid bij de verdediging. Ik kijk uit naar de discussies tijdens mijn verdediging!

Beste Willem en Marien, bedankt voor jullie waardevolle input en begeleiding. Vaak waren jullie de rots in de branding wanneer het echt even nodig was. Willem, je was als topexpert betrokken bij mijn project, maar eigenlijk was je een soort (RSV-)vader. Je kende iedereen binnen de virologie in Nederland, wist van alle (politieke) verhoudingen binnen het RSV veld en had altijd mooie verhalen over “vroeger”. Ik heb veel van je geleerd, over veel meer dan alleen RSV! Marien, ik ken weinig afdelingshoofden met zoveel hart voor de afdeling als jij. Je staat werkelijk voor iedereen klaar en maakt altijd tijd wanneer dat nodig is, ondanks je drukke agenda. Ik heb veel respect voor hoe je dat doet en vond het fijn dat ik altijd bij je terecht kon.

Lieve Sara en Hella, het is een fijn idee dat jullie straks naast mij staan tijdens de verdediging, maar ook daarna op de dansvloer! Sara, we zijn al buddy’s vanaf het begin van onze PhD projecten. Inhoudelijk zijn onze projecten erg verschillend, maar we wisten elkaar altijd te vinden als er dingen georganiseerd moesten worden of als we vonden dat er verandering nodig was op de afdeling. Ik ben nog steeds blij met ons vrijdagmiddagborrel-initiatief en ook al volg ik je goede raad niet altijd op (na 6 Leffe Blond richting Nieuwegein was écht wel een goed idee), ik vind het fijn dat we over alles kunnen praten samen! Hella, jouw spontane en attente persoonlijkheid is iets om trots op te zijn. Zonder dat je het zelf doorhad, heb je me door moeilijke tijden heen gesleept dankzij je positieve houding en lieve kaartjes. Ik hoop dat we, ondanks de afstand, wielren- en dansbuddy’s kunnen blijven!
Lieve kamergenootjes, wat heb ik veel aan jullie aanwezigheid gehad. **Daan, Elise en Sara**, we begonnen met zijn vieren in A12 en dat werkte zo goed dat we er alles aan hebben gedaan om samen te blijven na de verhuizing. Gelukkig lukte dat en kregen we er een IMS-baby bij: **Leon**. We hebben met zijn vijven veel gelachen en gehuild, en zonder jullie was mijn PhD traject een stuk frustrerender en eenzamer geweest. **Daan**, vooral het laatste jaar hebben we veel aan elkaar gehad. Je hebt geen makkelijk PhD traject, en de manier waarop jij je toch staande hebt weten te houden is super knap. Vergeet niet om wat vaker trots op jezelf te zijn en (kleine) overwinningen te vieren nu ik weg ben! **Elise**, je bent een groot voorbeeld voor me. De manier waarop jij je PhD zo “soepel” (op sommige vlakken) wist af te ronden, heeft ervoor gezorgd dat het mij ook is gelukt. Ik hoop dat we nog lang met elkaar lachen, zeuren, afreageren en toekomstplannen maken, ongeacht de afstand! **Leon**, ik ben blij dat we je geadopteerd hebben en weet zeker dat je goed terecht gaat komen, waar ook ter wereld! And last but not least: **Abi**. Gewoon in het Nederlands, want dat kun je “kei goed” (Southern dialect for “super goed”). We zijn maar kort roomies geweest, maar ik ben blij dat Elises plek door zo’n cool persoon is overgenomen die ook zo van dansen houdt: Keep on “shagging”!

Behalve mijn kamergenootjes, wil ik ook alle andere (oud) PhD’s (**Bette, Daantje, Elsbeth, Eric, Hella, Iris, Josien, Koen, Marieke, Marta, Michiel, Milou, Nora en Pauline**) bij IIV bedanken voor de gezellig barbecues, film- en spelletjesavonden. **Elsbeth**, ik heb heel veel respect hoe jij je project draaiende hebt gehouden de afgelopen jaren. Heel veel succes met de laatste loodjes van je PhD en de toelating voor je specialisatie! **Eric**, fitnessmaatje! Ik zal je bemoedigende high fives bij High Five missen. **Iris**, de ontwikkeling die jij hebt doorgemaakt de afgelopen jaren is echt ongelofelijk knap. Niet iedereen heeft het lef om de beslissingen te maken die jij hebt gemaakt. Blijf geloven in jezelf!

Ook op het lab heb ik de nodige hulp gehad; allereerst van de (master) studenten die ik heb begeleid tijdens mijn PhD project. **Maxime**, je was mijn eerste student en al vanaf dag 1 onmisbaar. Ik had namelijk net een nieuwe kruisband en in het lab werken op krukken is echt een uitdaging. Behalve op het lab, hadden we ook persoonlijk veel aan elkaar. Laten we onze Tony’s traditie in stand houden, ook als je straks een (drukkie) PhD.
plek hebt gevonden! **Mirjam**, jouw scriptie was geen makkelijke, maar het heeft de basis gelegd voor de prachtige review uit hoofdstuk 6, bedankt! En tot slot: **Anke**. Wat een doorzettingsvermogen heb jij laten zien tijdens je stage. Die ADCC assay was geen pretje, maar het heeft tot fantastische resultaten geleid die te lezen zijn in hoofdstuk 3. Ik ben heel blij dat je nog steeds bij ons in de groep werkt als analist en wellicht in de toekomst als PhD student. Ik vind het vaak moeilijk om dingen aan andere mensen over te laten, maar bij jou had ik er altijd vertrouwen in, en ik vind het dan ook heel fijn dat jij de huidige projecten helpt afronden. Bedankt voor al je toewijding en hulp de afgelopen jaren! Behalve studenten, hebben ook veel andere mensen me geholpen bij proeven die te ingewikkeld of te uitgebreid waren om alleen te doen. **Anke, Daan, Lie, Noortje, Puck en Teun**: bedankt voor jullie hulp op de vele sectiedagen. Zonder jullie hulp waren hoofdstuk 7 en 8 er nooit gekomen en hadden we de belangrijkste *in vivo* ontdekking van dit proefschrift nooit gedaan. **Ronald**, bedankt dat je mij in mijn eerste jaar wegwijst hebt gemaakt in de wereld van de PBMCs, het FACSen en de ADCC assay. Deze technieken vormen de basis voor mijn proefschrift. **Lie**, bedankt voor de vele virussen die je hebt gemaakt en waar ik altijd gebruik van mocht maken. **Rutger**, bedankt voor alle MIA's die jij voor ons hebt gedaan. Jouw aanpakmentaliteit maakt je een hele fijne samenwerkingspartner! **Sandra**, jij maakte het werk op het ML-II een stuk minder eenzaam, jammer dat we maar zo kort labmaatjes waren!

Dan zijn er ook nog een hele hoop mensen met wie ik niet inhoudelijk heb samengewerkt, maar die mijn tijd bij IIV heel wat leuker hebben gemaakt: **Diana** (je was een voorbeeld op vele fronten. Jouw betrokkenheid heeft me erg geholpen), **Harry** (altijd een lief gebaar op het juiste moment), **Karin** (topwijf ben je! De afdeling kan niet zonder je), **Rob v B** (bedankt voor alle leuke discussies en je interesse in mijn onderzoek), **Martijn**, **Rob M** en **Teun** (zonder jullie was de vrijdagmiddagborrel nooit zo'n succes geworden) en **Kina** (bedankt voor de lekkere baksels en decompenserende hardlooprandjes). Het liefst zou ik iedereen van centrum IIV hier persoonlijk noemen en bedanken, maar daar is helaas geen ruimte voor: dus ook als je niet genoemd bent, bedankt voor de hulp en gezelligheid de afgelopen vier jaar!
Al was ik meestal op het RIVM, ook op het Radboudumc lab was ik altijd welkom, iets waar ik vooral op feestjes en bij labuitjes gebruik van maakte. Dorien, we zijn samen begonnen en zijn nu allebei (bijna) klaar. Ik vind het leuk dat ons contact ook nog tot een mooie publicatie heeft geleid. Veel succes in Stanford! Marilen, we zijn van cord blood collega’s naar echte vriendinnen gegaan in korte tijd. Allebei flauwvallen op OK, onze gezamenlijke liefde voor bier, muziek en dansen, en allebei een vriend die altijd met klusprojecten bezig is: we hebben veel dingen gemeen. Succes met de laatste loodjes, ik geloof in je! Bryan, Esther, Evi en Lucille, ik kon altijd bij jullie terecht als dingen even tegenzaten en ik weer eens voor niks richting Nijmegen was gegaan. Ook als er iets op het lab moest gebeuren terwijl ik in Utrecht was, was dat geen probleem. Jullie hebben mijn gedeelde positie een stuk aangenamer gemaakt en waren gezellige congres- en retreat-buddy’s; bedankt! Christa, Elles, Fred, en Marc jullie hebben me wegwijs gemaakt op het lab in Nijmegen. Elles, ik vergeet nooit meer dat jij speciaal voor mij gedifferentieerde NHBE cellen ging maken. Super fijn dat jij je zo voor iedereen inzet, het heeft mij een hoop kopzorgen gescheeld! Fred, dankzij jou voelde ik me altijd welkom op de afdeling. Altijd een praatje en mooie vakantiekiekjes, en ook met mijn dierproef-frustraties kon ik bij je terecht: bedankt! Ook wil ik graag alle andere (oud-) LKI-ers bedanken: Dimitri, Erika, Inge A, Inge S, Jeroen, Jop, Josh, Kirsten, Koen, Ria en Saskia. Inge A, het cohort dat jij hebt verzameld vormt de basis voor twee hoofdstukken uit mijn PhD. Bedankt dat we daar gebruik van mochten maken! Inge S, ik vond het jammer dat je maar zo kort mijn RSV-maatje bent geweest, maar ik vind het ontzettend knap dat je je eigen pad bent gevolgd! Jop en Kirsten, onze ontmoeting op de dansvloer van mijn eerste PhD retreat heeft gezorgd voor een swingend begin van mijn PhD en zal ik nooit vergeten. Jop, ik heb veel van je geleerd op het lab, en vond het fijn dat ik ook altijd bij je terecht kon als ik een slaapplek in Nijmegen nodig had. Kirsten, ik heb altijd veel bewondering voor je gehad. Het is fijn om af en toe met je te kunnen sparren over de belangrijke dingen in het leven, let’s stay in touch!

A big thanks to all the people that were involved in my Master’s internship in St. Louis. Many thanks to Dr. Curiel for letting me experience all the sides of science during my internship. I enjoyed my time in the lab with Lyudmila and Sergey so much, that I decided to give the PhD–life a try. Lyudmila and
Sergey, many thanks for adopting me during my time in the US, I am glad to have a ‘Russian–American family’ on the other end of the world. En als ik het over St. Louis heb, dan moet ik jou natuurlijk ook onwijs bedanken: Sharon. Wat een avonturen hebben wij samen meegemaakt. Je bent een groot voorbeeld voor me (behalve als het over je omgang met beren gaat), en ik denk nog vaak met een lach terug aan onze St. Louis herinneringen.

Behalve collega’s zijn er ook een hoop vrienden die me hebben bijgestaan de afgelopen jaren. Laura, zonder jou was deze PhD een stuk moeilijker geweest. Je hebt me er vaak doorheen gesleept als ik weer eens gefrustreerd was over de voortgang van mijn PhD. Voor de buitenwereld lijken we zo verschillend als water en vuur, maar we vullen elkaar perfect aan als vriendinnen. Bedankt voor het samen lachen en samen huilen. Bedankt voor alle avonturen die we samen beleefd hebben en nog gaan beleven. Maar bovenal: bedankt dat je er altijd voor me bent.

Ook mijn rugbyvriendinnen van RUS verdienen een bijzonder plekje: De Fit old bosses, de Showpony’s, de meiden met wie ik (meerdere malen) het landskampioenschap heb mogen behalen en verdedigen: zonder jullie was ik niet wie ik nu ben. Aan rugby heb ik mijn discipline te danken, het heeft gezorgd dat ik tegen een stootje kan (zowel lichamelijk als mentaal), en daarbovenop had ik een flinke groep vriendinnen bij wie ik altijd terecht kon. De tijd bij RUS heeft me een van de belangrijkste motto’s uit mijn leven meegegeven: “Schaamte is een verspilling van je tijd” en daar hoop ik de rest van mijn leven in te kunnen geloven. Inmiddels heb ik er een tweede rugbyfamilie bijgekregen: RCN–ers, bedankt voor de liters bier en de gezelligheid zowel op als naast het veld!

Chantal, Erik, Guido, Joost, Manon, en Tom: Amigos! Wat bijzonder dat wij nog steeds zo’n goede vrienden zijn sinds het begin van onze studie. Het originele groepje 9 is wat uitgedund en aangevuld met groepje 10, maar na alles wat we overheerfd hebben (vooral dingen die hier niet genoemd hoeven te worden), hoop ik dat we nog vaak op avontuur gaan samen! Chantal, jou wil ik toch nog even extra bedanken, omdat je ook op het lab af en toe een reddende engel was: officieel werkte je niet voor onze groep, maar als ik een mega proef op de planning had, kon ik altijd op je rekenen: bedankt!
Iris, we hebben eigenlijk maar één jaar samen gehandbald, maar zijn daarna goede vriendinnen gebleven. Skeelertochtjes tussen Rosmalen en Heesch, een reis door Italië (“u wilt een Bacio?”), en tegenwoordig mijn Nijmeegse thuis als ik in het Radboud moest zijn. Bedankt dat ik altijd welkom ben! Remco, begonnen als wiskunde-maatjes in 4 VWO en nu allebei onze PhD bij het Radboud aan het afronden. We hebben ons eerste PhD salaris besteed aan de Tour de Mont Blanc, wat een van mijn tofste vakanties ooit zal blijven. Ik vond het fijn om altijd bij je terecht te kunnen voor een theetje op het Radboud of een biertje in kroeg, en vond jouw input tijdens onze discussies over mijn onderzoek altijd erg waardevol.

Ik wil ook heel graag de (oud-) IBB 15-IV burgerclub bedanken. Tijdens mijn studententijd én het begin van mijn PhD heb ik ontzettend veel aan jullie gehad. Na lange dagen op het lab, stond er altijd een heerlijke maaltijd klaar en dat maakte mijn eerste jaar als PhD een stuk aangenamer! Niks was te gek op 15-IV: van een jacuzzi op het balkon, tot pictionary spelen op de muren en het bouwen van een “man”-cave om samen Top Gear te kijken. Jullie zorgden altijd voor de nodige afleiding. Gelukkig wonen we (bijna allemaal) nog steeds binnen een straal van 3 km en kunnen we nog gewoon huiskamerkroegentochten doen. Marit, de serie-avondjes met onze vrienden Ben & Jerry zorgen altijd voor een welkom rustmomentje in mijn drukke schema. Ik ben blij dat we deze IBB-traditie als burgers hebben voortgezet!

En dan zijn we tot slot aangekomen bij mijn lieve (schoon)familie. Lieve van Erpjes: papa, mama, Kim en Philip. Jullie Brabantse nuchterheid zorgde ervoor dat ik dingen in perspectief kon plaatsen, terwijl jullie Brabantse gezelligheid (vaak juist niet nuchter) me hielp om het onderzoek af en toe even helemaal los te laten. Bedankt dat jullie er op alle belangrijke momenten voor me zijn. Jullie steun en vertrouwen betekenen veel voor me. Ook de rest van de familie van Erp en familie van Peufflik, ooms en tantes, nichten en neven, bedankt voor de nodige afleiding op familiedagen en verjaardagen en de interesse in mijn onderzoek. Mariska, grote nicht, super fijn dat je altijd zo geïnteresseerd was in mijn PhD project. Ik weet zeker dat je er inmiddels veel meer van snapt dan je zelf wilt toegeven en hoop dat je bij mijn verdediging kunt zijn! Hans en Grietje, Rudy en Neide, Anja en Aad: bedankt dat ik als een dochter/zus in jullie familie ben
opgenomen. De weekendjes Ardennen gaven vaak de nodige rust en afleiding. Anja, behalve een schoonzus, heb ik er met jou ook een goede vriendin bijgekregen. Ik hoop dat we nog vaak BB-dates, escaperooms en festivals meemaken in de toekomst!

Lieve Erwin, jij dacht dat je niet in mijn dankwoord hoorde, want “wat had jij nou bijgedragen aan mijn proefschrift?” Nou meneer, je hebt mijn promotietijd een stuk aangenamer gemaakt. Door jou begint iedere dag als een feestje, zelfs als ik daar om half 6 ‘s ochtends voor moet opstaan. Onze mooie reizen zorgden voor de nodige stressverlichting, al kreeg ik van jouw (klus)projectjes die stress soms dubbel en dwars weer terug. Maar dankzij al dat klussen wonen we nu wel in ons droomhuisje. Als experimenten weer eens mislukt waren door stomme foutjes of als ik gefrustreerd was over de langzame voortgang van het onderzoek, stond jij bij thuiskomst al met open armen (en een glaasje whisky) voor me klaar. Ja, jij hebt zeker bijgedragen aan dit mooie boekje als resultaat van mijn PhD: Dankzij jou heb ik het beste uit mezelf kunnen halen en daarvoor kan ik je niet genoeg bedanken. Ik hou van je!
THE ROLE OF MATERNAL ANTIBODIES IN RSV INFECTION
Emphasis on functionality and implications in disease