Respiratory Syncytial Virus

Anti-viral immunity in humans and macaques

Respiratoir syncytieel virus

Anti-virale immuniteit in mensen en makaken

Proefschrift

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De streep

Ik trok een streep,
tot hier,
nooit ga ik verder dan tot hier.

Toen ik verder ging
trok ik een nieuwe streep,
en nog een streep.

De zon scheen
en overal zag ik mensen,
haastig en ernstig,
en iedereen trok een streep,
iedereen ging verder

Toon Tellegen
Over liefde en over niets anders

Contents

Abbreviations

A (Ala)	alanine
Ab	antibody
ANOVA	analysis of variance
APC	antigen presenting cell
APC	allophycocyanin
APV	avian pneumovirus
ATCC	American type culture collection
aut	autologous
BAL	broncho-alveolar lavage
BB	albumin-binding domain of streptococcal protein G
BBG2Na	G2Na fused to BB
BLCL	B lymphoblastic cell line
BLCL-hRSV	B lymphoblastic cell line infected with hRSV
BPL	beta-propiolactone
bRSV	bovine respiratory syncytial virus
C (Cys)	cysteine
CBMC	cord blood mononuclear cells
CD	cluster of differentiation
CM	culture medium
cpts	cold-passaged temperature-sensitive
Ct	cycle threshold
CTL	cytotoxic T lymphocyte
D (Asp)	aspartic acid
DC	dendritic cell
DIF	direct immunofluorescence
DNA	desoxyribonucleic acid
E (Glu)	glutamic acid
ECACC	European collection of cell cultures
EDTA	ethylene-diamine-tetraacetic acid
E:T ratio	effector-to-target ratio
ELISA	enzyme-linked immunosorbent assay
ELISPOT	ELISA-based spot assay
Erasmus MC	Erasmus Medical Center
F (Phe)	phenylalanine
F	respiratory syncytial virus fusion protein
FACS	fluorescence activated cell scanner
FAM	6-carboxy-fluorescein
FBS	fetal bovine serum
FHA	family history of atopy
FI-bRSV	formalin-inactivated bovine respiratory syncytial virus
FI-hRSV	formalin-inactivated human respiratory syncytial virus
FITC	fluorescein isothiocyanate
FSC	forward scatter
G (Gly)	glycine
G	respiratory syncytial virus attachment protein
G2Na	bacterially-expressed central conserved domain of hRSV G ($G_{130-230}$)
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte/monocyte colony stimulating factor
GVHD	graft-versus-host disease
h	hour(s)
H (His)	histidine
HAI	haemagglutination inhibition

HI	heat-inactivated
HLA	human leukocyte antigen
hMPV	human metapneumovirus
HPS	human pooled serum
hRSV	human respiratory syncytial virus
hRSV-A	human respiratory syncytial virus subgroup A
hRSV-B	human respiratory syncytial virus subgroup B
I (Ile)	isoleucine
ICAM	intracellular adhesion molecule
ID	identification
IFN	interferon
Ig	immunoglobulin
IHW	international histocompatibility workshop
IL	interleukin
i.m.	intra-muscular
i.n.	intra-nasal
i.t.	intra-tracheal
IU	international units
K (Lys)	lysine
L (Leu)	leucine
L	respiratory syncytial virus major polymerase subunit
LRTI	lower respiratory tract infection
M (Met)	methionine
M	respiratory syncytial virus matrix protein
M2-1	respiratory syncytial virus anti-termination factor
M2-2	respiratory syncytial virus RNA regulatory protein
mAb	monoclonal antibody
MCP	monocyte chemotactic protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
MV	measles virus
MVA	modified vaccinia virus Ankara
N (Asn)	asparagine
N	respiratory syncytial virus nucleocapsid protein
NaCl	sodiumchloride
NBC	nasal brush cell
NBT/BCIP	nitro-blue-tetrazolium chloride / bromo-cloro-indolyl phosphate
NBTC	nasal brush T cell
NCBI	national center for biotechnology information
NIH	national institutes of health
nm	nanometer
NS1	respiratory syncytial virus nonstructural protein 1
NS2	respiratory syncytial virus nonstructural protein 2
nt	not tested
OD	optical density
OVA	ovalbumin
P (Pro)	proline
P	respiratory syncytial virus phosphoprotein
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridin chlorophyl protein
pfu	plaque-forming units

PHA	phytohaemagglutinin
PIV	parainfluenza virus
PVDF	polyvinylidene fluoride
PVM	pneumonia virus of mice
Q (Gln)	glutamine
R (Arg)	arginine
R10F	RPMI1640 supplemented with 10% fetal bovine serum
R10H	RPMI1640 supplemented with 10% human pooled serum
RANTES	regulated on activation, normal T cell expressed, and secreted
rhIL-2	recombinant human interleukin 2
RK-13	rabbit kidney cells
rMVA	recombinant modified vaccinia virus Ankara
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
rVV	recombinant vaccinia virus
S (Ser)	serine
SD	standard deviation
SFC	spot forming cells
SH	respiratory syncytial virus small hydrophobic protein
SPF	strictly pathogen free
SSC	side scatter
SSWO	Sophia foundation for medial research (in dutch: Sophia stichting voor wetenschappelijk onderzoek)
T (Thr)	threonine
TCC	T cell clone
$TCID_{50}$	tissue culture-infectious dose 50%
Th	T helper lymphocyte
Th0	T helper lymphocyte type 0
Th1	T helper lymphocyte type 1
Th2	T helper lymphocyte type 2
TMB	tetra-methyl benzidine
TNF	tumor necrosis factor
Triton X-100	polyethylene blycol tert-octylphenyl ether
Tween-20	polyoxyethylenesorbitan monolaurate
UNG	uracil DNA glycosylase
URTI	upper respiratory tract infection
UV	ultraviolet
V (Val)	valine
Vigall	virus-mediated allergy (in Dutch: virus-gemedieerde allergie)
VN	virus neutralizing
VV	vaccinia virus
W (Trp)	tryptophan
wt	wild-type
www	world-wide-web
Y (Tyr)	tyrosine

Chapter 1

General Introduction

1.1 Classification and characteristics

Human respiratory syncytial virus (hRSV) is a member of the family *Paramyxoviridae,* subfamily *Pneumovirinae*. This subfamily is divided in two genera: the genus *Pneumovirus* includes hRSV, bovine RSV (bRSV) and pneumonia virus of mice (PVM), the genus *Metapneumovirus* includes avian pneumovirus (APV) and tentatively also the human metapneumovirus (hMPV). hRSV is a single stranded negative-sense RNA virus, containing a non-segmented genome of 15.222 nucleotides encoding nine structural and two non-structural proteins [1]. The hRSV virion consists of a nucleocapsid contained within a lipid envelope and appears as irregular spherical particles ranging in diameter from 150 to 300 nm (figure 1). hRSV strains can be divided into two different subgroups, hRSV-A and -B, on basis of their reaction patterns with monoclonal antibody panels and nucleotide sequence differences between several of their genes [2-4].

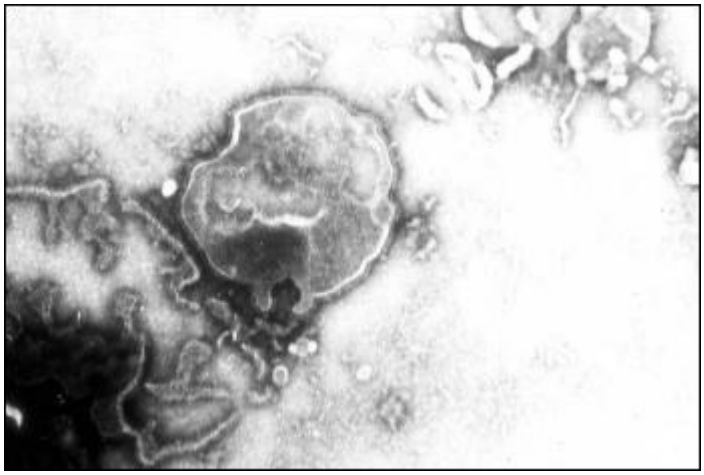

Figure 1: Negative contrast electron microscopic image of an hRSV virion.

The virus envelope contains three virus encoded transmembrane glycoproteins, the fusion (F) protein, the attachment (G) protein and the small hydrophobic (SH) protein. The F protein mediates fusion of the virus membrane with the host cell membrane, resulting in cell entry of the nucleocapsid. In addition, fusion of membranes of infected cells with those of neighboring cells can result in the formation of multinucleated syncytia [5]. The G protein is heavily glycosylated [6] and the ectodomain has, due to its high contents of serine, threonine and proline residues, structural similarities with mucins. It is involved in host cell attachment [7,8], and is thought to facilitate passage of the virus through the mucus-barrier [9]. The third envelope protein is the SH protein, of which the function remains unknown. Syncytium formation using plasmid-expressed F, G and SH was most efficient when all three proteins were present, suggesting a role for SH in enhancing the function of F and/or G [10]. However, both G and SH are

dispensable for hRSV replication in cell culture [8,11]. The matrix (M) protein, a nonglycosylated protein located on the inner side of the envelope, is thought to have two general functions: transcriptional inactivation of the nucleocapsid before packaging and association of the nucleocapsid with the newly developed envelope [1]. The major nucleocapsid (N) protein, the phosphoprotein P and the large polymerase subunit L are the viral proteins necessary and sufficient for RNA replication [12], with the anti-termination factor M2-1 being essential for viral viability [13]. The function of the nonstructural proteins NS1 and NS2 is largely unknown, but virions lacking one of these two proteins are attenuated *in vitro* and *in vivo* [14,15]. Finally, the M2-2 protein is a regulatory protein, which down-regulates transcription and up-regulates RNA replication [16].

1.2 Epidemiology and clinical manifestations

hRSV has a worldwide distribution and is one of the main causes of respiratory tract infections. It causes yearly epidemics in the winter season of moderate climate zones (figure 2) and in the rainy season of tropical climate zones [1]. Viruses of subgroup A or B can co-circulate during one epidemic, and epidemics may be dominated by either subtype. At three years of age all infants have been infected at least once, and re-infections continue to occur throughout life [17].

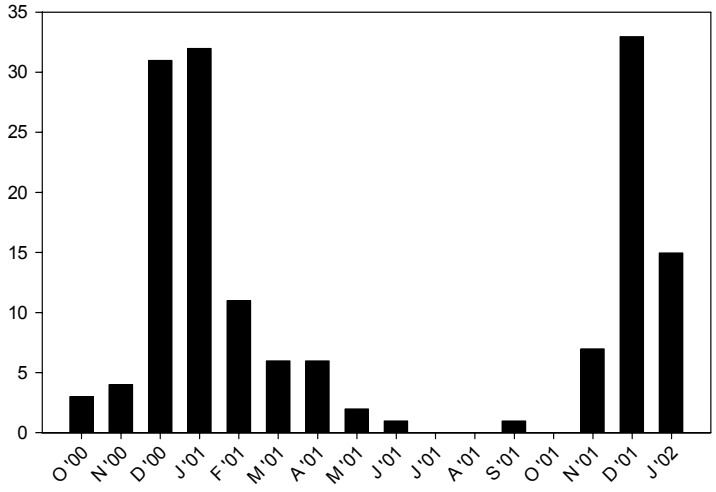

Figure 2: Monthly hRSV isolations as detected at the Department of Virology of the Erasmus MC Rotterdam from October 2000 to January 2002, illustrating the yearly winter outbreaks of RSV (taken from B.G. van den Hoogen *et al.* 2003 J Infect Dis **180**).

The incubation time from hRSV infection to onset of clinical signs is 4-5 days [1]. Relatively mild common cold-like upper respiratory disease can be seen in patients of all ages, but hRSV is also among the most important causes of severe lower respiratory tract disease, especially in preterm infants

[18], infants with underlying cardiac or respiratory disease [19,20], immunocompromised individuals and the elderly [17].

Clinical features seen during relatively mild hRSV infection are similar in all age groups and are generally limited to the upper respiratory tract [21]. Fever, irritating nonproductive cough and/or transient wheezing may be present, but the infection rarely leads to serious complications [22]. In severe hRSV disease the infection also involves the lower respiratory tract, patients may need mechanical ventilation, and in some cases outcome can be fatal. When compared with adults, the elderly have a higher risk (30-40%) for involvement of the lower respiratory tract upon hRSV infection, with rales and wheezing as the most common symptoms, but fever, cough and nasal discharge are also seen [23,24]. After hRSV infection in immunocompromised individuals upper respiratory tract infection precedes lower respiratory tract disease and acute lung injury [25]. Histopathology from autopsied cases has shown diffuse alveolar damage, severe squamous metaplasia, multinucleated giant cells, and intra-cytoplasmic inclusion bodies [25-27].

1.3 Immunity to hRSV

Host resistance to virus infections is mediated by both the innate and the adaptive immune response. Recent data suggest that early inflammatory and immune events play an important role in the outcome of acute hRSV infections. The importance of the adaptive immune response is illustrated by the failure of hRSV clearance in immunocompromised individuals [28,29]. Although complete protection from infection may not exist, hRSV-specific immunity can protect from severe lower respiratory tract disease.

1.3.1 Innate immunity

Upon infection of the local respiratory epithelium of the upper airways, the virus spreads along the respiratory tract. As a consequence, a number of molecules are produced by the epithelial cells, including potent immunomodulatory and inflammatory mediators such as cytokines (IL-1, TNF-α, IL-6 and IL-11), chemokines (IL-8, GRO, MCP-1, MIP-1α, RANTES), type I interferons (IFN-β/α) and growth factors (GM-CSF, G-CSF) [30,31]. In addition, type II alveolar epithelial cells are able to produce opsonins such as complement [32] and surfactant proteins [33] responsible for serum-independent phagocytosis of pathogens by neutrophils, monocytes and macrophages. Therefore, respiratory epithelial cells appear to be ideally located and armed to function as initiators of host defense mechanisms by regulating the prototypic cellular elements of the innate immune response, and may dictate the nature of the specific adaptive immune response to the virus [34].

Cytokines and chemokines produced upon infection can induce migration of eosinophils and neutrophils from the bloodstream into the infected tissue [35,36]. Neutrophils are the predominant airway leukocytes in hRSV bronchiolitis. In a study of 14 intubated infants they constituted 93%

and 76% of the inflammatory cells recovered from the upper and lower respiratory airways, respectively [37]. The cytotoxic effect of neutrophils is maximized by their retention at the site of infection. Neutrophils bind to ICAM-1, which is expressed on hRSV-infected respiratory epithelial cells [38], by a process that is both dose- and time-dependent [36,39]. As a consequence, prolonged presence of virus or viral antigens results in recruitment of more neutrophils to the airways.

Eosinophils can kill hRSV-infected cells by cytotoxic products, which are released by degranulation [40]. This degranulation can be activated by hRSV-infected cells in the appropriate environment of inflammatory mediators, but these infected cells can also activate uninfected epithelial cells, thus triggering further eosinophil degranulation [41]. Eosinophil-cytoxicity against bystander activated cells may in some cases contribute to pathogenesis of hRSV infection.

1.3.2 Adaptive immunity

Different compartments of the adaptive immune system are important in the immune response to hRSV. Virus-specific serum and secretory antibodies can neutralize the virus, and in very young infants maternally-derived specific antibodies are of importance [42]. The cellular immune response to hRSV includes hRSV-specific HLA class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTL) and HLA class II-restricted $CD4^+$ T helper (Th) lymphocytes. HLA class I molecules are present on almost all nucleated cells, and bind small (8-11 amino acids) peptides that are derived from proteins in the cytosol after proteolitic degradation [43-45]. As a consequence, virus-specific CTL usually recognize only infected cells. HLA class II molecules are present on specialized antigen presenting cells (APC) like dendritic cells (DC), macrophages and B cells, and bind longer (9-30 amino acids) peptides that are derived from proteins that are taken up from the extracellular environment by endocytosis [46]. As a consequence, virus-specific Th cells can recognize both infected APC and APC pulsed with inactivated antigen.

Both HLA class I and class II molecules are highly polymorphic, and a large number of alleles are present in the human population [47]. For both classes three genes exist: HLA-A, -B and -C for class I and HLA-DP, -DQ and -DR for class II. Depending on homo- or heterozygosity, each individual therefore carries three to six alleles of both classes. T cells recognize peptides that bind to one of these alleles, and are educated to discriminate between self and non-self HLA/peptide complexes, demonstrated by the crucial importance of HLA molecules in transplantation medicine [47].

1.3.2.1 Humoral immune response to hRSV

hRSV-specific virus neutralizing (VN) antibodies are present in the sera of all full-term newborns, due to the transplacental transfer of maternal antibodies [48]. Their levels decline during the first few months of life, and subsequently increase again as a consequence of natural infections with

hRSV [49,50]. In very young infants hRSV-specific antibody levels produced after infection usually remain low [51,52]. This limited response may be due to either the immaturity of the infant's immune system or the suppressive effect of maternally-derived antibodies [53]. At older ages, specific antibody titers rise after each hRSV infection, and although high levels of specific antibodies can be present in humans, these are usually not high enough to protect against infection [34]. Antibodies are developed to most of the structural proteins, but only antibodies specific for the F or G glycoprotein neutralize hRSV [1] and when transferred passively to animals only these antibodies confer protection [54]. It has been shown both in animal models and in infants that high levels of hRSV-specific VN antibodies protect against the development of severe lower respiratory tract infection [55,56], and reduce viral loads in infected premature infants [57,58].

1.3.2.2 Cellular immune response to hRSV

The importance of the cellular immune response to hRSV is illustrated by the absence of severe respiratory disease during early life in infants with agammaglobulinemia [59], in contrast to prolonged shedding of virus [28] and in some cases giant cell pneumonia [60,61] in individuals with a compromised cellular immune system. In addition, mice were shown to clear hRSV after adaptive transfer of specific $CD4^+$ or $CD8^+$ T cells [62].

Upon hRSV infection, both specific $CD4^+$ and $CD8^+$ T cell responses are generated, which can be directed to almost all proteins encoded by the hRSV genome. However, no MHC class I-restricted CTL responses specific for the hRSV G protein have been described in either mice or humans [63,64], but G-specific $CD4^+$ T cell responses are readily detectable in infected mice [65,66]. For humans, only a few hRSV-specific HLA class I-restricted CTL epitopes (table I), and no human HLA class II-restricted Th cell epitopes have been described.

Table I: HLA class I-restricted hRSV-specific T cell epitopes

Protein	Amino acid sequence	HLA restriction	Reference
F	R_{109}ELPRFMNYT_{118}	A*0101	[67]
N	N_{267}PKASLLSL_{275}	B7	[68]
N	V_{255}MLRWGVLA_{263}	B*08	[69]

Recently, van Bleek *et al.* [70] described the human CD4 response to the hRSV F protein using IFN-γ ELISPOT. By testing a set of overlapping peptides, the authors were able to demonstrate *ex vivo* F-specific CD4 memory T cell responses in peripheral blood mononuclear cells (PBMC) collected from adults, which were mostly restricted by HLA-DR and in a few cases by HLA-DQ. In this study epitope mapping was not performed. Previously, Levely *et al.* [71] demonstrated similar hRSV F-specific CD4 responses by proliferative assays.

In mice more T cell epitopes have been described: MHC class I-restricted epitopes have been identified for the hRSV F protein [72,73] and the M2 protein [74], while a class II-restricted epitope was described for the attachment G protein [66].

1.3.2.3 Mucosal immunity

The immune response to pathogens that enter the body at mucosal sites includes aspects of both the innate and the adaptive immune system [75]. Of the adaptive mucosal response to respiratory viruses, secretory IgA may be one of the most important mediators of protection to reinfection [76,77]. These molecules are actively transported to the mucosa and respiratory lumen, and may neutralize the virus before it is able to establish infection. In some studies in animals and humans a correlation was observed between hRSV-specific nasal IgA levels and protection [78]. However, due to the transient nature of specific IgA responses the protective effect is only short lasting.

1.4 hRSV pathogenesis and immunopathogenesis

Infections with hRSV are among the most important causes for severe lower respiratory tract disease in young infants, immunocompromised individuals and the elderly [17], and the virus can also cause influenza-like disease in adults [79,80]. However, the question remains why the majority of infections cause limited or no clinical signs, while a small subset of the infections is associated with severe disease. A first possible explanation is that some individuals could have a physiological predisposition, e.g. in the form of a low maternally-derived VN antibody titer or a pre-existent lung function abnormality [81]. Secondly, the infectious dose may be of importance [82], and also concurrent infection with other agents could result in enhancement of disease [83]. Finally, it has been hypothesized that the hRSV-specific immune response itself may contribute to the severity of the disease, or in other words that certain manifestations of hRSV-mediated disease are of immunopathological nature. The latter hypothesis is often associated with the hypothesis that hRSV-mediated lower respiratory tract disease during early life could induce, or be a marker for, an atopic asthmatic phenotype [84].

The immunopathology hypothesis to explain the pathogenesis of natural severe hRSV-mediated bronchiolitis should be clearly distinguished from the pathogenesis of hRSV vaccine-mediated enhanced disease. This was first observed in the 1960s, when infants vaccinated with a formalin-inactivated alum-precipitated whole virus preparation (FI-hRSV) were found to be predisposed for severe disease upon subsequent natural hRSV infection [85]. It is now generally accepted that the FI-hRSV-mediated enhanced disease had an immunopathological basis [86](see below).

1.4.1 Specific immune responses in infants with hRSV bronchiolitis

Information about the specific immune response during hRSV-mediated severe lower respiratory tract infection in humans is limited [87-89]. Infection with hRSV at a young age (between 6 weeks and 9 months of age) normally induces a cellular immune response associated with a combined Th1/Th2 (or Th0) cytokine profile. In some studies severe hRSV lower respiratory tract infections were found to be associated with a skewed Th2 response [87,89-94]. However, others have failed to confirm this observation [88,95-97].

1.4.2 A role for hRSV in the development of asthma or allergy?

Whether hRSV infection at a young age is involved in the development of atopic disease, like asthma and allergy, remains a subject of debate. Sigurs *et al.* found an association of severe hRSV infection at young age, subsequent episodes of wheezing and the development or exacerbation of asthma at the age of seven years [98], especially in infants with a family history of atopy. Also, Stein *et al.* showed an association between severe hRSV infection in infancy and wheezing in later childhood, but this association was absent by the age of thirteen years [99]. The potential role of mild hRSV infections in infancy in the development of atopic disease at later age is even more unclear. Forster *et al.* have shown that mild hRSV infections at a young age can promote aeroallergen sensitization during the first year of life, but they were unable to show atopic manifestations during the first two years of life [100].

It has been hypothesized that the putative relationship between hRSV and atopic disease has an immunological basis [101-106]. Studies in a mouse model of airway hyperresponsiveness have shown that hRSV challenge in ovalbumin (OVA)-sensitized mice enhances and prolongs airway inflammation and airway hyperresponsiveness, and that recurrent hRSV infections in sensitized mice shift the immune response toward Th2 immune responses, airway inflammation and airway hyperresponsiveness [107-109]. Studies in rodent models have shown that the production of IL-13, which is induced during hRSV infection in the airway, is a key factor in these processes [110-113]. IL-13 is also a key regulator in the pathogenesis of asthma [110]. In humans, IL-4 was described to play a role in episodes of wheezing [114], but whether IL-13, a cytokine also binding to the IL-4α receptor [115] and expressed by Th2 cells in patients with asthma, is also involved in this process remains to be determined [116,117].

1.4.3 hRSV vaccine-mediated enhanced disease in infants

Because of the high morbidity and mortality associated with hRSV infections, substantial efforts have been invested in the development of a vaccine [118,119]. However, a number of factors hamper the development of hRSV vaccines. Firstly, natural hRSV infections usually do not induce protective immunity [34,120], so the vaccine should induce more protective immune responses. Secondly, since a large part of the morbidity is seen in

very young infants, the vaccine should be able to mount an immune response at an early age and in the presence of maternally-derived antibodies [121]. Finally, vaccination trials with FI-hRSV in the 1960s resulted in enhanced disease upon subsequent natural hRSV infection in the next winter season [85,122]. Although the vaccine induced an antibody response to hRSV, this could not protect the infants. Instead, the infants showed a pulmonary hypersensitivity response, resulting in hospitalization of 80% of the youngest infants vaccinated and eventually the death of two of these infants [85]. Histopathological evaluation of sections of the lungs showed inflammation around the small airways, with infiltration of neutrophils, eosinophils and mononuclear cells [64,123]. The observations with FI-hRSV in infants and subsequent studies in laboratory animals have clearly illustrated that antigenic priming can result in either protective or disease-enhancing immune responses, depending on properties of the host, the immunizing agent and the route of administration [124].

1.4.4 hRSV vaccine-mediated enhanced disease in laboratory animals

Studies using murine models of hRSV infection have demonstrated that FI-hRSV is a strong inducer of Th2 cells, which seemed to be the most important mediators of the pulmonary pathology [86]. The non-replicating FI-hRSV vaccine does not induce HLA class I-restricted hRSV-specific CTL, which are not only crucial in clearance of infection but also produce IFN-γ which counteracts the development of a skewed Th2 response. The presence of alum in the vaccine further pushed the immune response to a skewed Th2 phenotype. This hypothesis was further supported by studies with recombinant vaccinia virus (rVV) expressing the F (rVV-F) or the G (rVV-G) gene of hRSV. If BALB/c mice were primed by dermal scarification with rVV-F and subsequently challenged with hRSV, normal immune responses with a balanced Th1/Th2 phenotype and no lung pathology were observed. However, when mice were primed with rVV-G they showed a skewed Th2 response associated with production of IL-4, IL-5 and IL-13, and pulmonary eosinophilia [125]. As mentioned before, the hRSV G protein could not be shown to induce CTL responses in BALB/c mice, thus mimicking the response induced by the FI-hRSV vaccine. Moreover, if mice were primed with an rVV-G in which a CTL epitope from the hRSV M2 protein was cloned, the induction of skewed Th2 responses and pulmonary eosinophilia was completely abolished [126]. The importance of $CD8^+$ CTLs was also illustrated by the observation that induction of a vigorous influenza virus-specific $CD8^+$ T cell response prior to sensitization with FI-hRSV prevented pulmonary eosinophilia after challenge with hRSV and attenuated the recruitment of inflammatory cells [127]. In a recent study in cynomolgus monkeys (*Macaca fascicularis*), FI-hRSV-mediated hypersensitivity to hRSV challenge proved to be associated with the induction of IL-13 producing Th2 cells after vaccination [128]. However, besides the specific cellular immune response, it has also been proposed that immune complexes

could play a role in the pathogenesis of hRSV vaccine-mediated enhanced disease [129].

1.5 hRSV vaccine development

Since the failure of the FI-hRSV vaccine in the 1960s, several studies have focused on the development of a live attenuated hRSV vaccine. However, it has generally been difficult to find a proper balance between attenuation and immunogenicity [130]. Whereas the initial strategy focused on the development of cold-passaged temperature-sensitive (cpts) mutant viruses, new technology has allowed the generation of mutant or chimeric viruses by using reverse genetics [130]. As an alternative for attenuated hRSV vaccines, live vectors mediating the expression of hRSV genes [131-136] as well as new generation non-replicating candidate hRSV vaccines have been considered [137,138]. A selection of the current approaches in hRSV vaccine formulation is shown in table II. Two of these vaccination approaches which were evaluated in macaques in the framework of the present thesis will be discussed in more detail below.

Table II: A selection of current approaches in hRSV vaccine formulation

Vaccine	Formulation	Reference
Subunit vaccines	purified F protein	[139,140]
	chimeric F / G protein	[141,142]
	bacterial-expressed central conserved domain of hRSV G fused to albumin-binding domain of streptococcal protein G (BBG2Na)	[137,143]
DNA encoding hRSV genes	F protein	[144,145]
	G protein	[146]
	mucosal delivery of DNA	[147]
Attenuated viruses	temperature-sensitive mutants	[148,149]
	gene deletion mutants	[150]
Genetically engineered viruses	chimeric hRSV-A / hRSV-B	[151]
	chimeric bovine / human parainfluenza type 3 expressing the hRSV F and G genes	[152]
	chimeric bRSV / hRSV	[153,154]
Recombinant live virus vectors expressing hRSV genes	rVV	[155]
	rMVA	[156]
	recombinant adenovirus	[132]
	recombinant vesicular stomatitis virus	[133]
	recombinant alphavirus	[134]

1.5.1 Subunit vaccine BBG2Na

BBG2Na is a subunit hRSV vaccine candidate based on a recombinant prokaryote-expressed protein [143]. It consists of the central conserved domain of the hRSV G protein (G2Na, amino acids 130-230) fused to the albumin-binding region of the streptococcal protein G (BB), and is formulated in alum. In different animal models it has been shown that vaccination with BBG2Na induced protective immunity to both subgroups of hRSV and no signs of FI-hRSV-like enhanced immunopathology was seen [143,157-160]. BBG2Na was also capable of inducing protective immunity in 1-week-old mice in the presence of high levels of hRSV A-specific maternal antibodies [161]. Evaluation of BBG2Na in a phase I/II study in healthy young adults showed that it was safe, well tolerated and immunogenic [137]. In a subsequent multi-center phase III study the vaccine was tested in the elderly, but results of this trial have not yet been made public. No standards are available for the pre-clinical information required before a candidate hRSV vaccine can proceed to clinical trials in seronegative infants, but the evaluation of efficacy and especially safety in this target group will be crucial also for all non-replicating hRSV vaccine candidates.

1.5.2 MVA as a vector for hRSV gene delivery

Modified vaccinia virus Ankara (MVA) is a replication-deficient poxvirus, which was used during the late stage of the smallpox eradication campaign. When used as a recombinant vector, rMVA induced similar levels of the inserted genes as compared to the fully replication-competent VV strains [162,163], and induced equal or better humoral and cellular immune responses in animals [164-166]. Since MVA is replication-deficient in most mammalian cells, it ensures safe usage of this vector, which was demonstrated in a safety study using immunocompromised macaques [167]. In addition, the vaccination dose can be relatively high as compared to replication-competent VV, which could contribute to overcoming the maternal antibody barrier. Replication-deficient vaccine vectors expressing hRSV genes represent attractive candidates for hRSV vaccine development. In the first place they can safely be used in preterm infants, immunocompromised patients or the elderly, who are all important target groups for a candidate hRSV vaccine. In addition, gene delivery using a viral vector would result in the *de novo* production of viral proteins by the vaccinee, resulting in presentation of hRSV-derived epitopes to both HLA class I- and class II-restricted T cells antigen and thus the induction of a balanced immune response.

1.6 Animal models in RSV research

Over the past decades a number of animal models have been used to reproduce hRSV vaccine-mediated enhanced disease, to study the pathogenesis and immunopathogenesis of this disease and to evaluate new candidate hRSV vaccines. Although few animal species have a similar susceptibility to hRSV infection as humans, studies performed in these

models can provide clues on how to improve current vaccines and vaccination strategies. In addition to hRSV models in different animal species, another interesting model is experimental infection with bRSV in calves, which allows pathogenesis studies in a natural host. However, hRSV disease is a multifaceted disease of which the clinical manifestations largely depend on age, genetic makeup and immunological status, both in humans and in animals. There is not a single human subpopulation in which all forms of hRSV disease are manifest, and equally there is no animal model, which can manifest all forms of hRSV pathogenesis or disease. Since different animal models have different strengths and weaknesses, the choice for a particular model will depend on the study objective in question.

1.6.1 Mice

The most extensively studied models for hRSV infection and pathogenesis are the mouse models, of which the BALB/c strain is the most often used [125]. The strength of this model is that it allows the study of hRSV in SPF inbred animals, for which a multitude of immunological backgrounds and reagents are available. Adoptive transfer studies can be performed, and the existence of many genetically modified or gene knockout strains allow evaluating the role of specific immunological molecules in the immune response to hRSV. Many studies of hRSV vaccine-mediated immunopathology were also performed in this species. However, a disadvantage is that the BALB/c mouse has a tendency towards Th2 responses, and several of the observations made in these animals could not be reproduced in other mouse strains [168]. Although it remains difficult to extrapolate results from mice to humans, these models can still help us to understand specific mechanisms involved in hRSV pathogenesis and immunopathogenesis.

1.6.2 Cotton rats

hRSV infection in cotton rats (*Sigmodon hispidus*) was first described by Dreizin *et al.* [169], and since cotton rats appeared to be about 100-fold more permissive than mice they seemed to be a good model to study hRSV infection and vaccine-mediated immunopathology [170,171]. Vaccination of cotton rats with FI-hRSV and subsequent hRSV infection indeed resulted in alveolitis and interstitial pneumonitis [172], and vaccination with new generation candidate vaccines resulted in protection from infection [141,143,173]. A weakness of the cotton rat model is the relative lack of reagents for characterization and quantification of the immune response, although these are currently being developed [174]. In addition, no congenic, transgenic or knockout cotton rats are available.

1.6.3 bRSV infection in calves

Human and bovine RSV are closely related viruses, and infections with these viruses in their respective hosts are associated with a similar pathogenesis. Infections with bRSV follow a seasonal pattern, and are the

major cause of respiratory disease in calves during the first year of life [175]. In addition, enhanced disease has been observed in animals vaccinated with inactivated bRSV vaccines after subsequent natural bRSV infection [176]. Experimental infections have been described in conventional and SPF calves, and the pathology of FI-bRSV-mediated enhanced disease could be reproduced [177,178]. However, characterization of specific immune responses in this species is difficult due to a relative lack of reagents.

1.6.3 Non-human primates

Monkeys are of special interest for the study of hRSV infection and immunopathogenesis, because of their close phylogenetic relationship to humans. However, only few species have a susceptibility to hRSV comparable to humans. The best model may be the chimpanzee (*Pan troglodytes*) [179], but for ethical, financial and other practical reasons it is difficult to use this species in an experimental animal model. Another species which was shown to have relatively good susceptibility to hRSV is the African green monkey (*Cercopithecus aethiops*), in which FI-hRSV-mediated immunopathology could be reproduced [180]. However, few immunological reagents are available for this species. Other monkey species used for experimental hRSV infection include owl monkeys (*Aotus trivirgarus*) [181,182], squirrel monkeys (*Samiri sciureus*) [131], rhesus monkeys (*Macaca mulatta*) [183], bonnet monkeys (*Macaca radiata*) [184,185] and cynomolgus monkeys (*Macaca fascicularis*) [128]. The latter three macaque species are of special interest, since many macaque-specific immunological reagents are currently available. A disadvantage of all non-human primate models is that ethical and financial constraints often limit the number of animals per group, which can result in statistically insignificant data. On the other hand, a range of clinical samples can be collected longitudinally, which may provide both qualitative and quantitative virological and immunological parameters of infection.

1.7 Outline of this thesis

Although T cells are considered to play a role in both protective and disease-enhancing immune responses, hRSV-specific T cell-mediated immunity is still poorly understood. In the present thesis hRSV-specific cellular immune responses were investigated in subjects with mild upper respiratory tract infections either or not caused by hRSV, and in patients with a severe hRSV bronchiolitis. In addition, the hRSV-specific T cell response was studied at a protein-specific and at a clonal level. Finally, the safety and efficacy of two new candidate hRSV vaccines were tested in a non-human primate model.

To study the role of the cellular immune response in the pathogenesis of hRSV-induced disease of different clinical severities, cytokine production by specific T cells obtained from different compartments was studied. In chapter 2, systemic (PBMC) and local (nasal) T cells were stimulated with autologous B-lymphoblastic cell lines (BLCL) either or not infected with

hRSV, and Th1 and Th2 cytokine producing cells were quantified in ELISPOT assays.

To study the hRSV-specific cellular immune response at the clonal level and to identify new T cell epitopes, T cell clones were generated. In Chapter 3 the identification of four new hRSV-specific T cell epitopes is described, two of which were restricted over HLA class I and the other two over HLA class II. In addition, hRSV-specific memory T cell responses to the hRSV F and G proteins were studied in rMVA-stimulated human PBMC bulk cultures.

In chapter 4, the evaluation of the safety and efficacy of two candidate new hRSV vaccines BBG2Na and rMVA-F/G (rMVA mediating the expression of the hRSV F and G genes) in a vaccination / challenge model in cynomolgus monkeys is described.

Finally, chapter 5 provides a summarizing discussion of the thesis.

Chapter 2

Moderate local and systemic respiratory syncytial virus-specific T cell responses upon mild or subclinical infection.

L. de Waal, L.P. Koopman, I.J. van Benten, A.H. Brandenburg, P.G.H. Mulder, R.L. de Swart, W.J. Fokkens, H.J. Neijens, A.D.M.E. Osterhaus

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Abstract

Human respiratory syncytial virus (hRSV) infections are a major cause of severe respiratory disease in infants. It has been shown that there is an increased frequency of childhood wheezing in ex-bronchiolitic preteen children. This was postulated to be mediated by a vigorous virus-specific Th2 response influencing the further development of the immune system. Little is known about the possible role of the immune response to clinically mild hRSV infections in this respect. We have studied the hRSV-specific cellular immune response in infants with a laboratory-confirmed hRSV upper respiratory tract infection (URTI; *n*=13, mean age 12 months, range 2-22 months) in comparison with infants with non-hRSV mediated URTI (*n*=9, mean age 9.3 months, range 4-18 months) or infants with severe hRSV bronchiolitis (*n*=11, mean age 2.3 months, range 1-6 months). hRSV-specific cytokine producing cells were enumerated using the ELISPOT method in peripheral blood mononuclear cells and nasal brush T cells, collected during the acute and convalescent phase of the infection. Mixed Th1 (IFN-γ) and Th2 (IL-4 and IL-13) responses were detected in all three groups. Frequencies of hRSV-specific T cells were lower in both URTI groups than in the hRSV bronchiolitis group, and not significantly different between the hRSV URTI and the non-hRSV URTI group. The absence of vigorous virus-specific Th2 responses upon mild hRSV infection does not support the hypothesis that these infections influence the development of the immune system and that they predispose for the development of atopic disease.

Introduction

Human respiratory syncytial virus (hRSV) infections are the most important cause of severe respiratory disease in infants below 6 months of age. In particular, preterm infants [18] and infants with underlying cardiac and respiratory disease [19,20] have an increased risk to develop severe lower respiratory tract disease upon hRSV infection. Although most often associated with relatively mild upper respiratory tract infections (URTI) in immunocompetent adults, the virus may also cause severe respiratory disease in immunocompromised individuals and the elderly [17]. In moderate climate zones, hRSV causes yearly epidemics during the winter season, and at the age of three all infants have been infected at least once; reinfections continue to occur throughout life [17].

At present, no licensed hRSV vaccine is available, although several approaches are being exploited [118]. hRSV vaccine development remains hampered severely by vaccine trials in the 1960s when a formalin-inactivated whole virus preparation (FI-hRSV) was found to predispose infants for enhanced clinical disease upon subsequent natural hRSV infection [85,122,186,187]. Although the exact mechanism of this apparently immunopathological phenomenon remains unclear, studies in rodent models of hRSV infection have demonstrated that priming for a Th2 cellular immune

response is a key factor in this hypersensitivity response [86,188-190]. Studies in rodent models have also illustrated that not only FI-hRSV but also priming with the G protein of hRSV alone resulted in similar hypersensitivity responses upon subsequent hRSV infection, again associated with predominant Th2 responses [126,191-193].

Although several studies have suggested that primary hRSV infections in infants resulting in severe hRSV bronchiolitis are also associated with Th2 cellular immune responses [87,89], others have been unable to confirm this observation. Brandenburg *et al.* [88] found a predominant Th1 cellular immune response regardless of clinical severity.

Severe hRSV infection at a young age has been associated with episodes of wheezing and the development or exacerbations of asthma at the age of seven years, especially in infants with a family history of atopy [98]. In addition, Stein *et al.* [99] showed a relationship between severe hRSV infection in infancy and wheezing in later childhood, but this relationship was lost by the age of 13 years. Whether this relationship is causal or whether these phenomena result from a common physiological predisposition continues to be a subject of debate [194,195]. Because hRSV bronchiolitis and atopic disease have both been associated with Th2 cellular immune responses and rises in specific IgE, it has been hypothesized that the putative relationship between hRSV and asthma or allergy has an immunopathological basis [101-106]. Studies in a mouse model of airway hyperresponsiveness have shown that hRSV challenge in OVA-sensitized mice enhances and prolongs airway inflammation and airway hyperresponsiveness [107,196]. Recurrent hRSV infections in allergen-sensitized mice shift the immune response even further toward Th2 immune responses, airway inflammation and airway hyperresponsiveness [108].

A key factor in these processes of wheezing and airway hyperresponsiveness seems to be the production of the Th2 cytokine IL-13. Studies in rodent models of airway hyperresponsiveness have shown that IL-13 is the main mediator of the exacerbation of allergic response during hRSV infection. Moreover, IL-13 is induced preferentially during hRSV infection in the airway, and promotes airway hyperresponsiveness and damage [110-113,197]. In humans the Th2 cytokine IL-4 has been described to be involved in episodes of wheezing after hRSV-induced bronchiolitis [103]. Whether IL-13 also plays an important role in the induction of airway remodelling, wheezing and airway hyperresponsiveness in humans, as seen in rodent models of airway hyperresponsiveness after hRSV infection, remains to be proven.

Despite many studies investigating the role of hRSV bronchiolitis in the development of asthma or allergy, the putative role of hRSV-specific cellular immune responses in infants with relatively mild hRSV-induced URTI (RSV URTI) remains unclear. We selected infants with mild hRSV URTI, non-hRSV URTI or severe hRSV bronchiolitis, and characterized both the systemic and local hRSV-specific cellular immune responses. To this end, Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokine producing cells were

enumerated in peripheral blood mononuclear cells and T cell lines obtained from nasal brush samples following stimulation with autologous hRSV-infected B-lymphoblastic cell lines.

Materials & Methods

Patients and study design

Samples were collected in the framework of two different projects, both approved by the medical ethical committee of the Erasmus MC in Rotterdam. Informed consent was obtained from the parents. The first project was a prospective birth cohort study (Vigall) in which 129 healthy infants with (*n*=89) or without (*n*=40) FHA were followed until 2 years of age. Clinical specimens were collected at 6, 12, 18 and 24 months of age (routine visits), and during the acute and convalescent (approximately 14 days later) phases of each URTI, as defined by rhinorrhoea and at least one of the following criteria: fever, general malaise or loss of appetite. From this cohort 13 infants were selected with a laboratory-confirmed hRSV URTI (mean age 12 months, range 2-22 months) and 9 infants with a non-hRSV URTI (mean age 9.3 months, range 4-18 months). Patients with a non-hRSV URTI were selected based on the inclusion criteria mentioned above and a negative diagnosis for hRSV. In the second project (SSWO), clinical specimens were collected during the acute and convalescent (approximately 28 days later) phase from 11 infants hospitalized with severe hRSV-related bronchiolitis (mean age 2.3 months, range 1-6 months). Clinical samples included a heparinized peripheral blood sample and a nasal brush sample (Cytobrush Plus; Medscand Medical AB, Tomelilla, Sweden). Patient characteristics and viral diagnostics are mentioned in table I.

Plasma was isolated and stored at -70°C. Peripheral blood mononuclear cells (PBMC) were collected by density gradient centrifugation and stored at -135°C. Nasal brush cells (NBC) were harvested within 4 hr after sample collection in RPMI1640 medium (BioWhittaker, Verviers, Belgium) containing gentamycin by scrubbing the brush along the side of the tube and centrifuging for 5 min at 400 x *g*.

Laboratory diagnosis of hRSV infections

hRSV infections were diagnosed by combining the results of direct immunofluorescence (DIF) using fluorescein isothiocyanate (FITC)-labeled hRSV-specific monoclonal antibodies (DAKO, Glostrup, Denmark) on nasal brush cells [52] and virus isolation from nasal brush supernatant on HEp-2 cells.

Cell lines

Autologous B-lymphoblastic cell lines (BLCL) were established by transformation of PBMC with Epstein-Barr virus as described previously [198]. For the generation of target and stimulation cells, these BLCL were infected with hRSV A2 (multiplicity of infection [moi]: 100; ATCC-

VR1322) resulting in persistently hRSV-infected BLCL as described previously [199]. The percentage of hRSV-positive cells was checked by fluorescence activated cell scanner (FACScan) analysis with FITC-labeled hRSV-specific monoclonal antibody (DAKO). All BLCL were maintained in

Table I: Patient characteristics

Patient	Gender (M/F)	FHA (Y/N)	Age at onset (mo)	Month	Infectious agent	Symbol in figures 2-4	Fill
Group 1: hRSV URTI							
1	F	Y	20	Nov	hRSV	□	Gray
2	M	Y	16	Feb	hRSV	○	Gray
3	M	Y	12	Nov	hRSV	△	Gray
4	M	Y	22	Apr	hRSV	▽	Gray
5	F	Y	6	Jan	hRSV	◇	Gray
6	F	Y	10	Apr	hRSV	⬡	Gray
7	M	Y	8	Feb	hRSV	□	None
8	F	Y	7	Dec	hRSV	○	None
9	F	Y	15	Nov	hRSV	△	None
10	M	Y	18	Dec	hRSV	▽	None
11	F	Y	2	Nov	hRSV	◇	None
12	M	N	16	Jan	hRSV	⬡	None
13	F	N	4	Jan	hRSV	⊕	None
Group 2: non-hRSV URTI							
1	M	Y	5	Jan	ND	□	Gray
2	M	Y	6	Jan	rhino/corona	○	Gray
3	M	Y	7	Dec	ND	△	Gray
4	M	Y	18	Apr	corona	▽	Gray
5	F	N	4	Dec	rhino	◇	Gray
6	F	Y	18	Apr	ND	⬡	Gray
7	F	Y	11	Apr	ND	□	None
8	M	Y	11	Mar	ND	○	None
9	F	N	4	Nov	rhino	△	None
Group 3: hRSV bronchiolitis							
1	F	N	1	Jan	hRSV	□	Gray
2	F	Y	3	Jan	hRSV	○	Gray
3	M	N	1	Feb	hRSV	△	Gray
4	M	Y	1	Mar	hRSV	▽	Gray
5	F	N	3	Dec	hRSV	◇	Gray
6	F	N	1	Feb	hRSV	⬡	Gray
7	M	Y	3	Dec	hRSV	□	None
8	F	N	2	Dec	hRSV	○	None
9	M	N	2	Dec	hRSV	△	None
10	M	N	6	Feb	hRSV	▽	None
11	F	Y	2	Feb	hRSV	◇	None

M, male; F, female; FHA, family history of atopy; mo, months; ND, not determined

RPMI1640 medium (BioWhittaker) containing penicillin (100 U/ml; BioWhittaker), streptomycin (100 µg/ml; BioWhittaker), L-glutamin (2mM; BioWhittaker), and β-mercapto-ethanol (10^{-5}M; Merck KGaA, Darmstadt, Germany), further referred to as culture medium (CM), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Greiner, Frickenhausen, Germany).
Within 24 hr after sample collection, nasal brush T cells (NBTC) were stimulated with phytohaemagglutinin (PHA) and gamma-irradiated allogeneic feeder cells and expanded with recombinant human IL-2 (rhIL2; Eurocetus, Amsterdam, the Netherlands) as described previously [200] in CM supplemented with 10% heat-inactivated human pooled serum (R10H). After 2-4 weeks of expansion, these NBTC were stored at -135°C.

ELISPOT assay

Two weeks before the ELISPOT assay, PBMC and NBTC were thawed, stimulated with PHA and gamma-irradiated allogeneic feeder cells, and expanded for 2 weeks in R10H supplemented with rhIL-2.

From the expanded PBMC, $CD8^+$ cells were isolated by positive selection with magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's instructions, whereas remaining PBMC were tested as $CD8^-$ (mainly $CD4^+$) cells. NBTC were tested without prior separation in $CD8^+$ and $CD8^-$ cells. Cells were stimulated first with autologous hRSV-infected BLCL (effector-to-target ratio [E:T] of 2), autologous uninfected BLCL (E:T of 2) or R10H for 1.5 hr at 37°C in 96-wells V-bottom plates (Greiner). For IFN-γ 10,000-15,000 effector cells were used per well, whereas 100,000 effector cells were used for IL-4 and IL-13. Subsequently, the cells were transferred to ELISPOT plates with either a nylon bottom (for IFN-γ; Nalge Nunc, Rochester, NY, USA) or with a PVDF bottom (for IL-4 and IL-13; Millipore, Molsheim, France), which had been coated overnight at 4°C with a monoclonal antibody against IFN-γ (7.5 µg/ml; Mabtech AB, Stockholm, Sweden), IL-4 (15 µg/ml; Mabtech AB) or IL-13 (15 µg/ml; Mabtech AB) and blocked with R10H for 2 hr at 37°C. After 3-4 hours of incubation at 37°C, plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-T; Merck KGaA, Darmstadt, Germany) and incubated overnight at 4°C with a secondary biotinylated antibody against IFN-γ (0.5 µg/ml; Mabtech AB), IL-4 (1 µg/ml; Mabtech AB) or IL-13 (1 µg/ml; Mabtech AB). Spots were visualized using streptavidine alkaline phosphatase (1:1,000; Mabtech AB) for 2 hr at room temperature, washing with PBS-T and adding NBT/BCIP substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) until spots appeared. Finally, washing the ELISPOT plates with distilled water stopped the color reaction. Spots were counted using an automated ELISPOT reader (Automated ELISA-Spot Assay Video Analysis Systems; A.EL.VIS GmbH, Hanover, Germany) for IFN-γ spots, or a stereomicroscope at 25-fold magnification for IL-4 and IL-13 spots. The number of hRSV-specific spots was determined by subtracting the number of spots detected after stimulation with uninfected BLCL from

the number of spots detected after stimulation with hRSV-infected BLCL (represented per 10^6 cells).

hRSV serology

An optimized "in-house" ELISA was used to determine hRSV-specific total IgG in plasma samples collected during routine sampling moments (at 6, 12, 18 or 24 months of age). In short, medium binding ELISA plates (Greiner) were coated overnight with 100 µl purified whole hRSV-A antigen at a concentration of 1 µg/ml in PBS at room temperature and washed three times with 300 µl PBS containing 0.5% Tween-20 (Merck KGaA). Plasma samples, diluted 1:100 in ELISA buffer (Meddens Diagnostics, Vorden, The Netherlands) supplemented with 5% normal goat serum (ICN, Irvine, CA, USA), were added and plates were incubated for 1 hr at 37°C. Next, plates were washed and 100 µl horseradish peroxidase-labeled goat anti-human IgG (Biosource, Camarillo, CA, USA) was added in ELISA buffer for 1 hr at 37°C. Finally, 100 µl TMB-substrate (Meddens Diagnostics) was added and staining reaction was stopped by addition of 2 M H_2SO_4. The extinction was measured at 450 nm with a reference filter at 620 nm.

Statistical analysis

Data were analyzed using nonparametrical tests. Differences between the two sampling moments (acute and convalescent) in each group were analyzed using the paired Wilcoxon test. Differences between the non-hRSV URTI and the hRSV URTI group during the acute or convalescent phase were analyzed using the Mann-Whitney test. Differences were considered statistically significant if the p-value < 0.05.

Results

Patient characteristics

Within the cohort of 129 infants followed up during their first two years of life (Vigall), 80 URTI episodes were reported, of which 13 were diagnosed as acute hRSV infections. An additional seven acute hRSV infections were diagnosed during routine sampling moments in the absence of clinical symptoms. Because a larger number of hRSV cases had been expected, prevalence of hRSV-specific IgG antibodies was measured in a number of plasma samples collected at the ages of 6 (n=54), 12 (n=49), 18 (n=21) and 24 months (n=26). Figure 1 shows the kinetics of plasma IgG antibodies to hRSV during this 2-year period. After a decline during the first year due to the loss of maternal antibodies, six of 21 samples (76%) were positive at 18 months and 22 of 26 samples (85%) were positive at 24 months of age. These data confirm that the majority of infants in the cohort had experienced at least one hRSV infection during their first 2 years of life. In most cases, these infections apparently did not result in URTI as defined in the Materials & Methods section, were not reported to the study doctor or were not diagnosed properly. Twenty laboratory-confirmed acute hRSV cases were identified and

13 were selected on basis of sample availability for characterization of hRSV-specific cellular immune responses. Of these 13 infants, eight were selected during the acute phase of an hRSV infection and five during a routine sampling moment. These were compared to responses in infants from the same cohort study with an URTI not related to an acute hRSV infection (*n*=9) and with responses in infants hospitalized with a severe hRSV-related bronchiolitis (*n*=11).

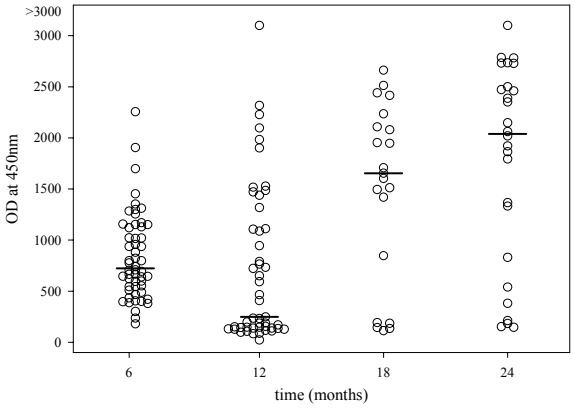

Figure 1: Detection of RSV-specific IgG during the different routine sampling moments. Results are shown for each individual infant and each routine sampling moment. The median of each group is represented by a line.

hRSV-specific cytokine producing T cells

ELISPOT assays were used to enumerate hRSV-specific Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4 and IL-13) producing T cells in the infants mentioned above. To compare systemic hRSV-specific T cell responses to those at the primary site of infection, NBTC were also tested. These NBTC were tested without separation of $CD8^+$ and $CD8^-$ T cells, whereas PBMC were tested after separation of $CD8^+$ and $CD8^-$ T cells. Furthermore, these hRSV-specific cellular immune responses were examined both during the acute and convalescent phase of infection.

Before and after expansion the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were measured by FACScan analysis. Mean percentages (± SD) of these cells in PBMC (p) or NBTC (n) in the acute (1) or convalescent (2) phase are shown in table II. Results show that the mean percentage of $CD4^+$ T cells did not change during expansion, whereas the percentages of $CD8^+$ T cells rose during this expansion. Note that for NBTC, these are the percentages after the first and second aspecific expansion cycle.

hRSV-specific T cell responses in severe hRSV-related bronchiolitis

In infants with severe hRSV-related bronchiolitis, IFN-γ producing T cells could be detected in the $CD8^+$ fraction of PBMC and in NBTC during both the acute and convalescent phase of infection. In the $CD8^-$ fraction of PBMC, IFN-γ producing T cells could only be detected during the convalescent phase of infection (figure 2). In contrast, in some infants Th2 cytokine (IL-4 and IL-13) producing T cells could be detected in the $CD8^-$

fraction of PBMC during both the acute and convalescent phase of infection, whereas in the $CD8^+$ fraction of PBMC and in NBTC, these cells could only be detected during the convalescent phase of infection (figure 3 and 4). Overall, the frequencies of hRSV-specific T cells, either Th1 or Th2, in infants with severe hRSV-related bronchiolitis were higher during the convalescent phase than the acute phase of infection.

These data confirm that our assays were of sufficiently sensitivity to detect hRSV-specific T cells producing Th1 and Th2 cytokines.

Table II: $CD3^+CD8^+$ and $CD3^+CD4^+$ cells in PBMC or NBTC

	before aspecific expansion		after aspecific expansion	
	$CD8^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells	$CD4^+$ T cells
p1	15.2% (7.4)	50.9% (13.8)	36.8% (17.2)	51.2% (19.9)
p2	15.6% (6.4)	52.2% (14.1)	39.7% (14.7)	43.4% (14.2)
n1	50.6% (22.8)	18.8% (17.9)	64.1% (22.9)	18.3% (16.4)
n2	66.4% (21.3)	11.5% (11.9)	70.5% (20.6)	16.4% (15.3)

Mean % (SD) of cells collected during the acute (p1, n1) or convalescent (p2, n2) phase, measured before and after aspecific expansion. p, PBMC; n, NBTC.

RSV-specific T cell responses in hRSV vs. non-hRSV URTI

In infants with hRSV URTI as well as in infants with non-hRSV URTI, relatively low numbers of IFN-γ producing T cells (figure 2) and virtually no IL-4 and IL-13 producing T cells (figure 3 and 4) were detected in the $CD8^+$ or $CD8^-$ fraction of PBMC collected during the acute or convalescent phase of the infection. In NBTC from these infants, IFN-γ producing T cells could be detected during both the acute and convalescent phase of the infection (figure 2). Although a few individuals in the hRSV URTI group showed higher frequencies of hRSV-specific T cells, this did not result in a significant difference between the two groups. In NBTC from infants with non-hRSV URTI no IL-4- or IL-13 producing T cells could be detected, whereas in some infants with hRSV URTI these T cells were detected (figure 3 and 4).

Discussion

hRSV-specific T cell responses were studied in infants with mild hRSV URTI, non-hRSV URTI and severe hRSV bronchiolitis. Frequencies of hRSV-specific T cells were lower in both URTI groups as compared to the hRSV bronchiolitis group, but not significantly different between the hRSV or non-hRSV URTI groups. No substantial qualitative differences in the phenotype of the cellular immune response were found between the three groups.

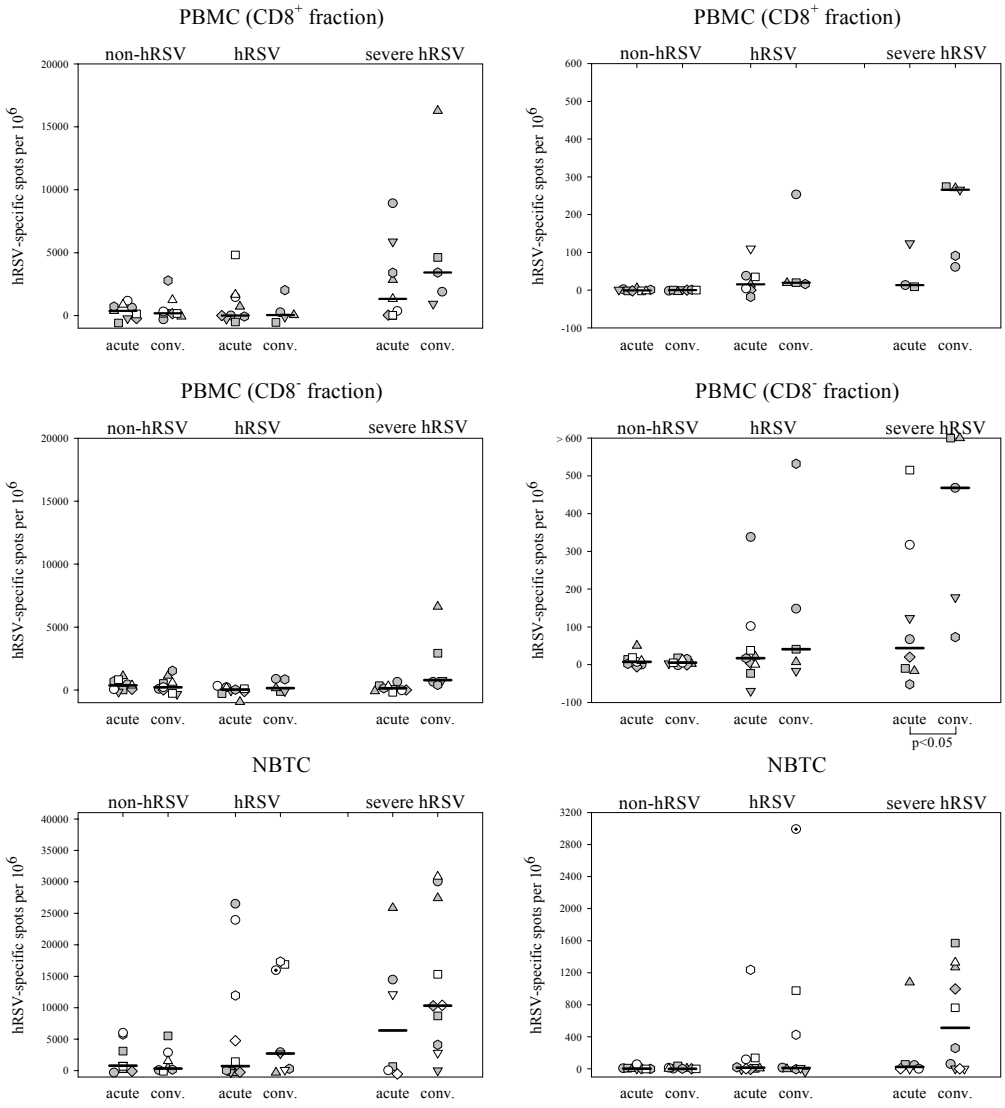

Figure 2: Detection of hRSV-specific IFN-γ producing T cells per 10^6 within the $CD8^+$ fraction of PBMC (top graph), the $CD8^-$ fraction of PBMC (middle graph) and the NBTC (bottom graph) during the acute and convalescent phase of infection. Each graph represents the number of hRSV-specific IFN-γ producing T cells for non-hRSV URTI (non-hRSV; left), hRSV URTI (hRSV; middle) and severe hRSV bronchiolitis (severe hRSV; right). The median of each group is represented by a line. Note the differences in the scales of the figures for the different populations tested in the ELISPOT assay.

Figure 3: Detection of hRSV-specific IL-4 producing T cells per 10^6 within the $CD8^+$ fraction of PBMC (top graph), the $CD8^-$ fraction of PBMC (middle graph) and the NBTC (bottom graph) during the acute and convalescent phase of infection. Each graph represents the number of hRSV-specific IL-4 producing T cells for non-hRSV URTI (non-hRSV; left), hRSV URTI (hRSV; middle) and severe hRSV bronchiolitis (severe hRSV; right). The median of each group is represented by a line. Note the differences in the scales of the figures for the different populations tested in the ELISPOT assay.

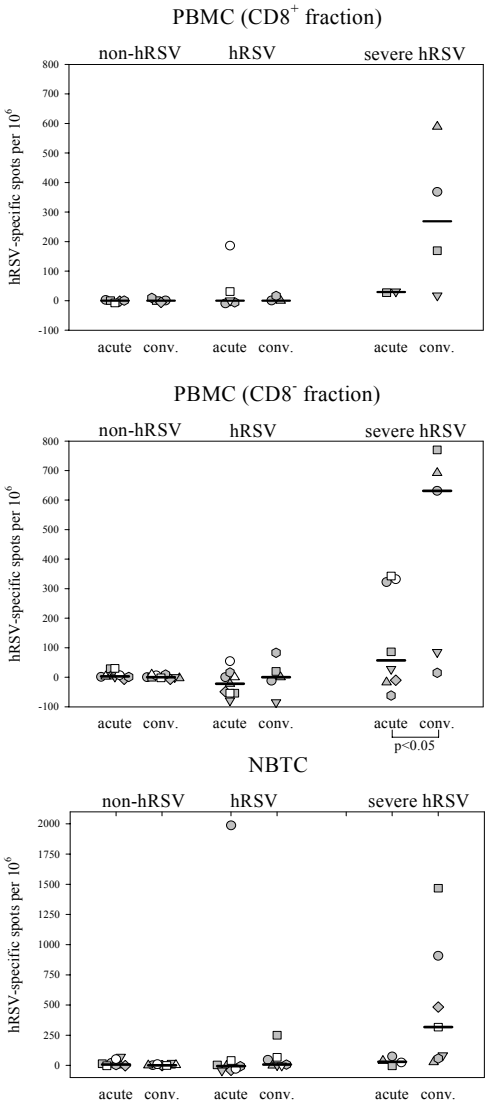

Figure 4: Detection of hRSV-specific IL-13 producing T cells per 10^6 within the $CD8^+$ fraction of PBMC (top graph), the $CD8^-$ fraction of PBMC (middle graph) and the NBTC (bottom graph) during the acute and convalescent phase of infection. Each graph represents the number of hRSV-specific IL-13 producing T cells for non-hRSV URTI (non-hRSV; left), hRSV URTI (hRSV; middle) and severe hRSV bronchiolitis (severe hRSV; right). The median of each group is represented by a line. Note the differences in the scales of the figures for the different populations tested in the ELISPOT assay.

We set out to test the hypothesis that infants with FHA would be predisposed to respond to hRSV infections during early childhood with a skewed virus-specific Th2 response. Because we detected only a limited number of acute hRSV infections in our prospective birth cohort study, however, which were found predominantly in the group of infants with FHA (table I), it was decided to limit our objectives to the characterization of the hRSV-specific T cell response in infants with mild or subclinical hRSV infections.

We identified no more than 20 laboratory-confirmed hRSV cases in 129 healthy infants followed during their first 2 years of life, of which 13 were associated with mild URTI and seven were found during routine sampling of clinically healthy infants. The prevalence of hRSV-specific serum IgG antibodies in infants' samples at the age of 18 or 24 months indicated that the majority of these infants had experienced at least one hRSV infection during their first 2 years of life, in accordance with other cohort studies [49,50]. Based on the literature, it was expected that few primary hRSV infections would be asymptomatic [1], most infections would be associated with URTI and 25-40% with hRSV bronchiolitis [201-203]. In our cohort study, two patients suffered from hRSV-induced bronchiolitis as described by others [202]. The limited number of URTI episodes reported (n=80 for 129 infants over a 2-year period) suggests underreporting in our study.

When comparing the three patient groups, the frequencies of hRSV-specific cytokine producing cells were significantly higher in hRSV

bronchiolitis patients than in URTI patients. The differences in age and timing of the convalescent phase sample in the URTI and the severe hRSV bronchiolitis groups, however, must be taken into consideration. Therefore, no firm conclusions may be drawn from these results, but it could be seen as a trend. Cells, both systemically and locally, producing the Th1 cytokine IFN-γ were measured at frequencies one to two log-values higher than cells producing the Th2 cytokines IL-4 or IL-13. Brandenburg *et al.* [88] previously studied hRSV-specific T cell responses in infants with hRSV infections of different clinical severity by specifically stimulating PBMC with autologous hRSV-infected BLCL, followed by expansion with rhIL-2, and subsequent characterization of the expanded cultures by measurement of cytokine production. In this study, it was found that responses were dominated by IFN-γ producing T cells, although IL-4 and IL-10 producing T cells could be detected at low frequencies. A difference was not detected in this respect between infants with severe or mild bronchiolitis. The results of our present study suggest that use of the ELISPOT assay on cells expanded with a nonspecific protocol (PHA + allogeneic feeder cells) is a more sensitive method for this purpose. We cannot rule out that the use of the expansion and cryopreservation steps introduces selection of certain subsets of T cells or changes in sensitivity/specificity. The data of both studies, however, suggest that, although low levels of Th2 cytokines or cytokine producing cells can be detected, the hRSV-specific T cell response is dominated by cells of the Th1 phenotype. The IFN-γ response detected in NBTC from infants with non-hRSV URTI can more easily be attributed to the presence of an hRSV-specific T cell response generated during an earlier hRSV infection.

Several studies have investigated the phenotype of the hRSV-specific T cell response in different patient groups. Aberle *et al.* [93] showed that infants suffering from severe hRSV infection had lower levels of IFN-γ mRNA expression in PBMC compared to infants with mild hRSV infection. Similar results were obtained by Bont *et al.* [204], who tested cytokine levels in nasal aspirates. They showed that ventilated infants had lower levels of IFN-γ in nasal aspirates than non-ventilated infants. Roman *et al.* [87] showed that infants suffering from hRSV-LRTI not only had decreased levels of IFN-γ in PHA-stimulated PBMC, but that the levels of IL-4 were decreased to a lesser extent, indicating a more Th2-like immune response. In contrast, our data suggest a more quantitative than qualitative difference in the hRSV-specific cellular response between patients with different disease severity. It is interesting to note that in our study IL-4 and IL-13 producing cells, rather than IFN-γ producing cells, were detected at higher levels in convalescent than in acute samples. This suggests a T cell phenotype switch over time as described previously for measles virus-specific T cells [205]. Our results are also in accordance with the data presented by Pala *et al.* [91], who showed that ex-bronchiolitic patients at the age of 7-8 had higher frequencies of hRSV-specific IL-4 producing T cells than control children. In this study, the frequencies of hRSV-specific IFN-γ producing T cells did not

differ between the two groups; however, the stimulation protocols were fundamentally different: Pala *et al.* used irradiated hRSV resulting in the stimulation of $CD4^+$ T cells only, whereas we used hRSV-infected autologous BLCL resulting in the stimulation of both $CD4^+$ and $CD8^+$ T cells.

When seeking a link between hRSV infections and the development or exacerbation of allergic asthma, IL-13 producing T cells may be of special interest, because this cytokine has been shown to play a central role in the pathogenesis of asthma and atopic disease [110,206]. IL-13 has been identified previously as a predominant cytokine produced by hRSV-specific T cells upon vaccination of macaques with FI-hRSV [128].

It has been suggested that in some cases, hRSV could establish a persistent infection in the host [207]. In addition, hRSV is known to cause recurrent infections throughout life, which could result in a chronic stimulation of hRSV-specific T cells. In a recent study, hRSV genome could be detected by RT-PCR in the lungs of three of seven adults who died of an asthma death and five of seven asthmatic patients who died of unrelated causes, as opposed to none of the seven control individuals [208]. We detected IL-13 producing cells predominantly in bronchiolitis patients during the convalescent phase. If a phenotype switch from Th1 to a mixed Th1/Th2 response indeed occurs after a primary hRSV infection, continuous re-stimulation could result in an increase of the frequency of hRSV-specific IL-13 producing cells. It could be hypothesized that boosting of IL-13 producing hRSV-specific T cells contributes to the pathogenesis of asthma. This hypothesis, however, could be tested best by prospectively comparing hRSV-specific cellular immune responses between infants with or without FHA and subsequently determining which of these children develop asthma or atopic disease.

In conclusion, our data suggest that asymptomatic primary hRSV infections are more common than considered previously. The hRSV-specific T cell response during mild or subclinical hRSV infections was similar to that observed in non-hRSV patients. On the other hand, the hRSV-specific T cell response in infants with hRSV bronchiolitis was higher quantitatively when compared to infants with hRSV URTI. It is unlikely that the immune response in infants with hRSV URTI could be a factor in driving the development of the immune system in the direction of skewed Th2 responses. The demonstration of hRSV-specific IL-13 producing T cells in hRSV bronchiolitis patients during the convalescent phase, however, suggests an alternative explanation for the observed link between bronchiolitis during early childhood and wheezing or development of atopic disease at a later age.

Acknowledgements

We thank all children and parents participating in both studies, and H. Timmerman and B. van ‘t Land for technical assistance in processing the clinical specimens. These studies were financially supported by The Netherlands Asthma Foundation, The Netherlands Organization for Health Science and the Sophia Foundation for Medical Research. We received an additional research grant from the Foundation ‘Vereniging Trustfonds Erasmus Universiteit Rotterdam’ in The Netherlands.

Chapter 3.1

HLA class I-restricted cytotoxic T cell epitopes of the respiratory syncytial virus fusion protein.

A.H. Brandenburg, L. de Waal, H.H. Timmerman, P. Hoogerhout, R.L. de Swart, A.D.M.E. Osterhaus

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Summary

Virus-specific cytotoxic T lymphocytes (CTL) play a major role in the clearance of human respiratory syncytial virus (hRSV) infection. We have generated cytotoxic T cell clones (TCC) from two infants who had just recovered from severe hRSV infection. These TCC were functionally characterized and used to identify HLA class I (B57 and C12)-restricted CTL epitopes of hRSV.

hRSV is a common cause of upper respiratory tract infections but may - especially in young infants, the elderly, or immunocompromised individuals - cause severe lower respiratory tract infections [1]. In rodent models, $CD4^+$, as well as $CD8^+$, hRSV-specific T lymphocytes proved to be involved in both recovery from and immunopathogenesis of the infection [125,192,209,210]. Therefore, a fine balance must exist in these models between protective and disease-enhancing effects of virus-specific T lymphocytes. hRSV-specific $CD8^+$ cytotoxic T lymphocytes (CTL) against virtually all hRSV proteins have been demonstrated to circulate in humans after hRSV infection [211-213]. CTL may be expected to play a crucial role in the clearance of hRSV infections, but their role in protection and immunopathology remains unclear. Therefore, the identification of CTL epitopes of hRSV may contribute to future studies concerning the role of CTL in pathogenesis and protection from hRSV infection. Here we describe the functional characterization of $CD8^+$ cytotoxic TCC generated from two infants who had just recovered from severe hRSV infection. These TCC were also used to identify, for the first time, HLA class I-restricted CTL epitopes of hRSV.

Peripheral blood mononuclear cells (PBMC) were collected from two infants, 4 weeks after a severe, laboratory-confirmed hRSV infection. At the time of infection, they were 1 and 2 months old and had both been admitted to the intensive care unit. B-lymphoblastic cell lines (BLCL) were generated by Epstein-Barr virus transformation [198], infected with hRSV A2 (ATCC VR1322), and UV irradiated [214] to serve as autologous antigen-presenting cells (APC). PBMC (3×10^4/well in 96-well round-bottom plates) were stimulated with APC (10^4/well), and expanded in RPMI1640 medium supplemented with antibiotics, 10% pooled and heat-inactivated human serum, and recombinant human interleukin-2 (rhIL-2; 50 IU/ml). After 2 weeks, T cells were harvested and cloned by limiting dilution using phytohaemagglutinin stimulation as previously described [200]. TCC thus generated were expanded and tested for hRSV specificity by ^{3}H-thymidine incorporation assays as previously described [198]. All of the hRSV-specific TCC proved to be of the $CD8^+$ phenotype in fluorescence-activated cell scanner (FACScan) analysis. TCC whose specificity for hRSV was confirmed by a second proliferation assay were tested for protein specificity by an interferon-gamma (IFN-γ) ELISPOT assay (Mabtech AB, Stockholm, Sweden). In this test, paraformaldehyde-fixed autologous BLCL that had

been infected with recombinant vaccinia viruses (rVV) expressing different hRSV proteins (F, G, N, P, M2, SH, M, 1B, or 1C) were used as APC. Briefly, TCC (5 x 10^3/well) were incubated with APC (10^4/well) for 4 h, transferred to anti-human IFN-γ-coated plates, and incubated for another 18 h. The ELISPOT assay was further performed in accordance to the kit manufacturer's instructions. The results are shown as numbers of IFN-γ producing cells (spots) per well.

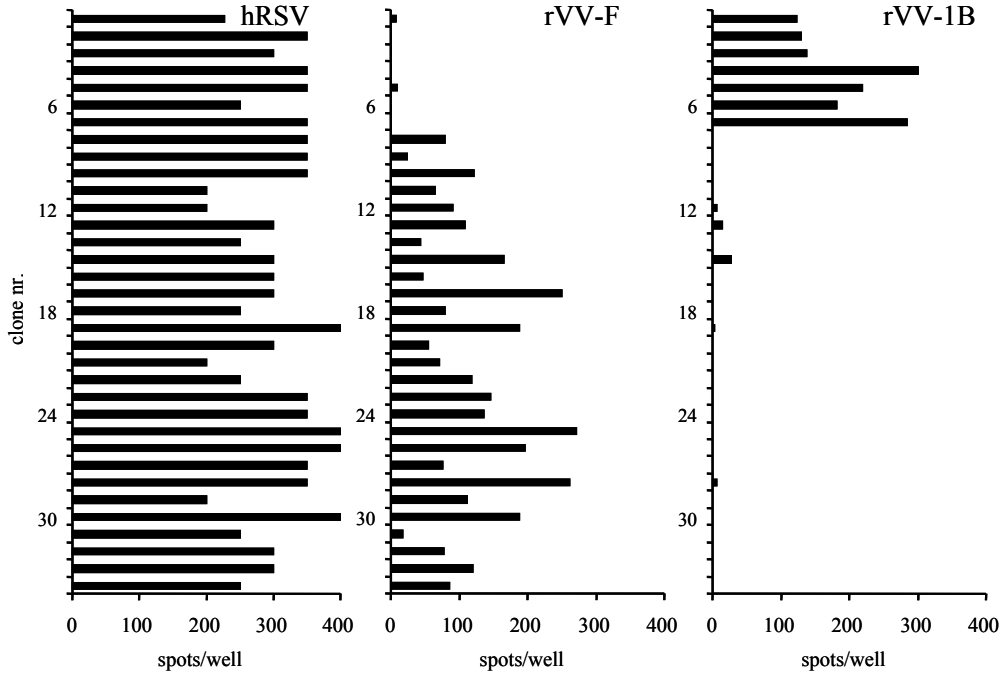

Figure 1A: Responses of hRSV-specific TCC from patient 1 measured by an IFN-γ ELISPOT assay using autologous BLCL infeted with hRSV A2 (left), rVV expressing the hRSV F (middle) or 1B (right) proteins. Results are indicated as numbers of spots per well. No responses against the BLCL infected with the other rVV were found (data not shown).

Thirty-four hRSV-specific TCC were generated from the PBMC of patient 1, as detected in a ^{3}H-thymidine incorporation assay. Of these, 27 proved to be hRSV F specific and 7 were hRSV 1B specific in an IFN-γ ELISPOT assay (figure 1A). Twenty-four hRSV-specific TCC were generated from the PBMC of patient 2 as detected in a ^{3}H-thymidine incorporation assay. Of these, 10 were hRSV F specific and 14 proved to be hRSV 1C specific in an IFN-γ ELISPOT assay (figure 1B). None of the clones detected by the ^{3}H-thymidine incorporation assay was found to be negative by the IFN-γ ELISPOT assay. Since the F protein is considered to be a major CTL target [211-213], further efforts were focused on the identification of CTL epitopes in the F protein. To this end, 18-mer peptide

amides overlapping by 12 amino acids, together spanning the entire F protein of hRSV A2 [215,216](*n* = 94), were generated in an automated multiple-peptide synthesizer as previously described [217]. The purity of the peptides varied between 50 and 90%, as determined by analytical reverse-phase high-performance liquid chromatography (C_8 column; gradient of 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile). Autologous BLCL of patients 1 and 2 were pulsed overnight with 1 and 3 µM

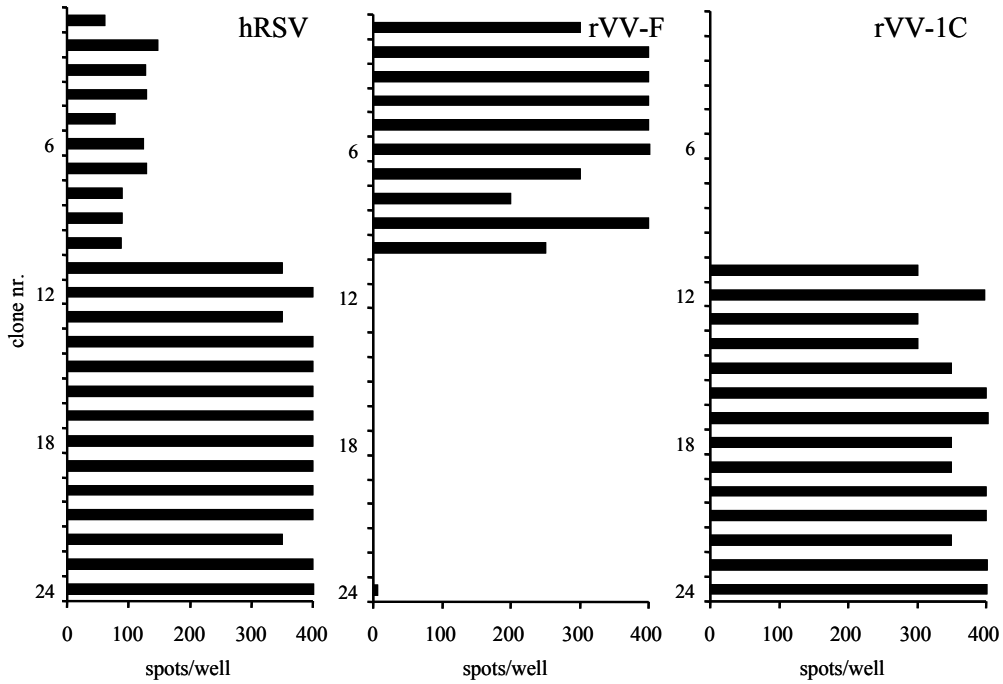

Figure 1B: Responses of hRSV-specific TCC from patient 2 measured by an IFN-γ ELISPOT assay using autologous BLCL infected with hRSV A2 (left), or rVV expressing the hRSV F (middle) or 1C (right) proteins. Results are indicated as numbers of spots per well. No responses against the BLCL infected with the other rVV were found (data not shown).

peptide, respectively. HRSV F-specific TCC of patient 1 reacted with peptides 17 and 18 (figure 2A). All of the hRSV F TCC of patient 2 reacted with peptide 91 and marginally with peptide 90 (figure 2B). Subsequently, two additional sets of partially overlapping 8- to 12-mer peptides (80 to 95% purity) were generated to determine the respective minimal epitopes. Autologous BLCL of patients 1 and 2 were pulsed for 1 h with the different peptides at 1 and 3 µM, respectively. All of the hRSV F-specific TCC of patient 1 reacted with one nine-mer peptide (RARRELPRF) spanning residues 118 to 126 of the F protein (figure 2C). The hRSV F TCC of patient 2 all reacted with one nine-mer peptide (IAVGLLLYC) spanning residues 551 to 559 of the F protein (figure 2D).

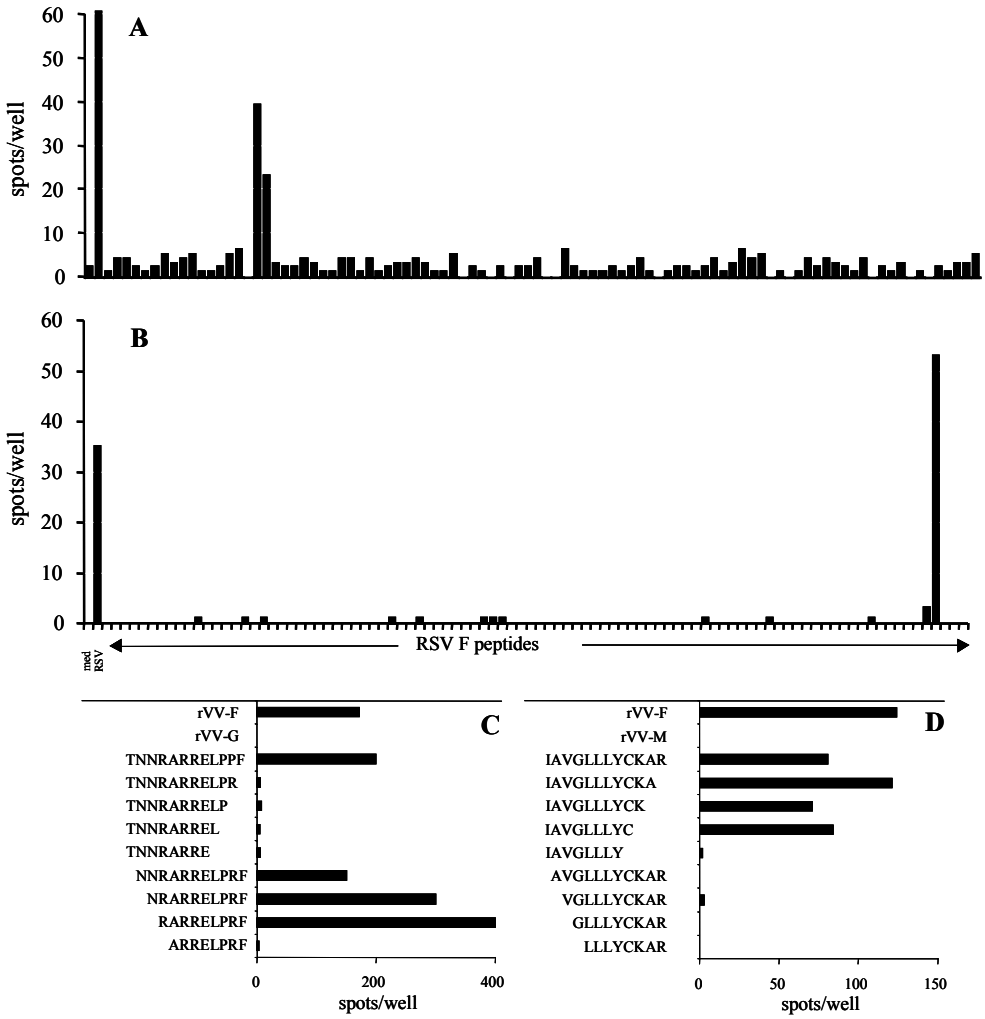

Figure 2: Fine mapping of T cell epitopes on the hRSV F protein. Responsiveness of TCC from patients 1 (A) and 2 (B) to partially overlapping 18-mer peptide amides (*n*=94) spanning the F protein was measured by an IFN-γ ELISPOT assay. Minimal peptide activation of TCC from patients 1 (C) and 2 (D) was measured in an IFN-γ ELISPOT assay using APC pulsed with two additional sets of overlapping 8- to 12-mer peptides. med, medium.

The HLA restriction of the recognition by the hRSV F TCC was also determined by the IFN-γ ELISPOT assay using a set of allogeneic BLCL with partially matched HLA types loaded with the respective minimal peptides, as shown in figure 3. Recognition of the hRSV F epitope of patient 1 proved to be HLA B57 restricted. Recognition of the epitope of patient 2 proved to be C12 restricted.

Well-growing TCC of both specificities from each of the patients were arbitrarily selected and tested for cytotoxic activity by a chromium release assay as previously described [218]. Briefly, autologous ^{51}Cr-labeled, hRSV-

infected BLCL and control BLCL or, in the case of the F-specific TCC of patient 2, minimal-peptide-loaded BLCL or control BLCL were incubated with TCC for 4 h at 37°C at an effector-to-target cell ratio of 10:1, which was found to be the most discriminative ratio in preliminary experiments. Spontaneous ^{51}Cr release (target cells plus medium) and maximum ^{51}Cr release (target cells plus Triton X-100) were determined in 12 identical wells. Supernatants were harvested and analyzed in a gamma counter. Assay results were accepted only when the spontaneous-to-maximum release ratio was <25%. All of the TCC tested showed hRSV-specific lysis (figure 4A). Virus-specific production of cytokines in cell-free culture supernatant was measured as previously described [88]. TCC were stimulated with hRSV-infected BLCL or control BLCL or, in the case of the F-specific TCC of patient 2, minimal-peptide-loaded BLCL or control BLCL for 48 h. The concentrations of the following cytokines were measured using commercially available sandwich enzyme-linked immunosorbent assay systems in accordance with the manufacturers' instructions: IL-4 (CLB, Amsterdam, The Netherlands; detection limit, 7 pg/ml), IFN-γ (Medgenix Diagnostics, Fleurs, Belgium; detection limit, 25 pg/ml), IL-10 (Pharmingen, San Diego, CA, USA; detection limit, 20 pg/ml). All of the TCC produced predominantly IFN-γ, indicating a type 1 phenotype *in vitro* (figure 4B).

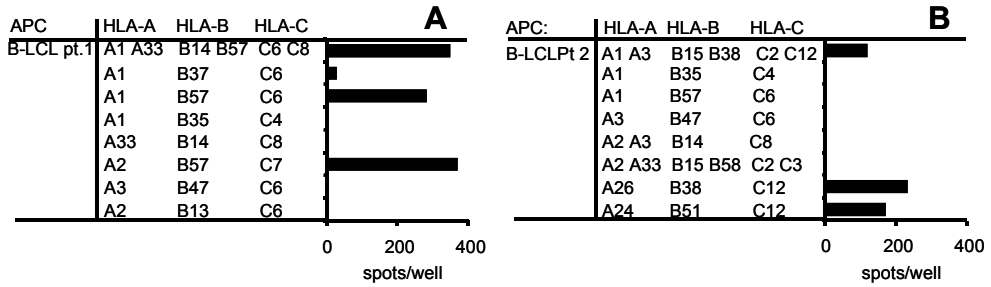

Figure 3: Determination of HLA restriction of hRSV F protein recognition by specific TCC using a set of autologous and partially HLA class I-matched BLCL for patients 1 (A) and 2 (B) by an IFN-γ ELISPOT assay.

In the present study, we identified two nine-mer CTL epitopes on the hRSV fusion protein. To our knowledge, these are the first HLA class I-restricted CTL epitopes described for hRSV in humans. We found CTL epitopes with an HLA restriction with a low prevalence in the population. But using the techniques described here on samples of more children with a recent hRSV infection, CTL epitopes with more prevalent HLA restrictions may be identified.

The TCC found in the children studied were all $CD8^+$ CTL with a type 1-like cytokine profile. No hRSV-specific $CD4^+$ T cells were detected in the samples of these infants, while the use of similar stimulation protocols did result in the identification of such cells in other systems [198,219-221]. Virus-specific CTL responses play a major role in the clearance of hRSV

infections but may also be involved in pathogenesis [61,192,199,209,222]. In mice, enhanced lung pathology induced by priming with formalin inactivated hRSV has been associated with the absence of a CTL response [126,189]. In young children, a CTL response against hRSV has been described but poor responses were found in younger and more severely ill patients [223,224]. The poor CTL response in young children has been suggested to be one of the possible causes of more severe disease. In these two patients, it was possible to detect hRSV-specific cytotoxic TCC, showing that also in very young children with clinically severe infections, priming of a CTL response does occur, although we cannot say anything about the quantitative responses.

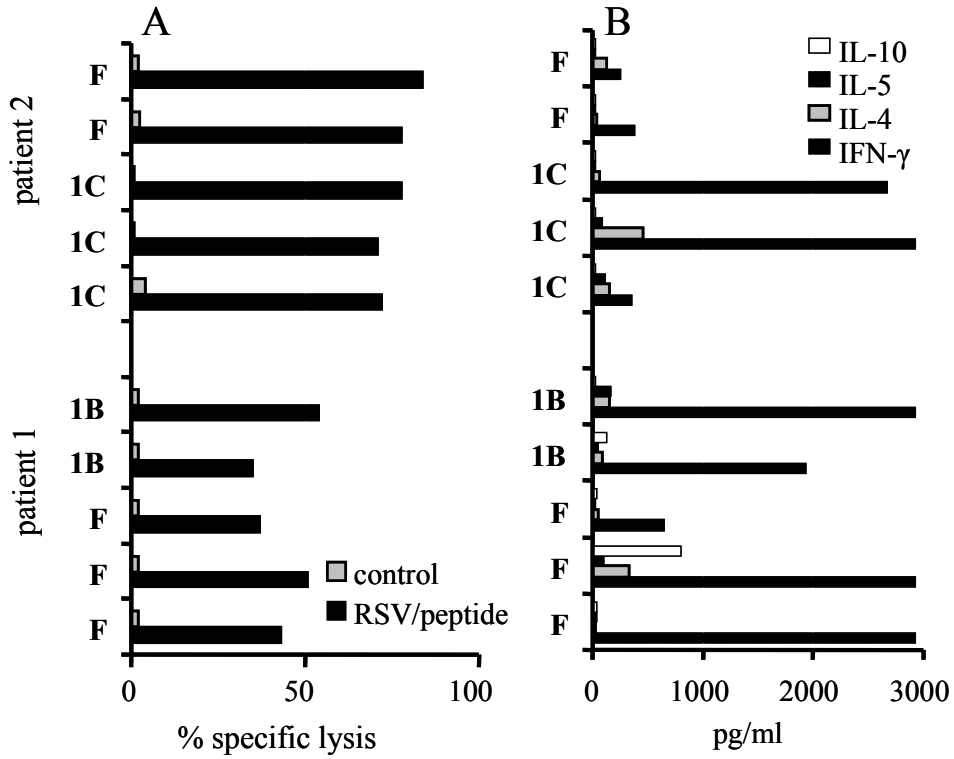

Figure 4: Cytotoxic responses (A) and cytokine production (B) of selected TCC with different protein specificities from patients 1 and 2. TCC were stimulated with autologous hRSV-infected or control BLCL and in the case of F-specific TCC of patient 2, minimal-peptide- or control (measles virus) peptide-loaded BLCL. Cytotoxic respones were measured in a chromium release assay using an effector-to-target cell ratio of 10. Cytokines in cell-free culture supernatant (IFN-γ, IL-4, IL-5 and IL-10) were measured by enzyme-linked immunosorbent assay. Levels of cytokines in the supernatant of TCC stimulated with control BLCL were all below the detection limit and are not shown.

In conclusion, use of the IFN-γ ELISPOT assay to determine epitope specificity proved to be sensitive and convenient, since only small numbers of T cells and APC were needed. This and similar studies may be important for future studies concerning the role of CTL in the pathogenesis of hRSV infection in children.

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Chapter 3.2

Identification of a common HLA-DP4-restricted T cell epitope in the conserved region of the respiratory syncytial virus G protein.

L. de Waal, S. Yüksel, A.H. Brandenburg, J.P.M. Langedijk, K. Sintnicolaas, G.M.G.M. Verjans, A.D.M.E. Osterhaus, R.L. de Swart

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Abstract

The cellular immune response to hRSV is important in both protection and immunopathogenesis. In contrast to HLA class I, class II-restricted hRSV-specific T cell epitopes have not been identified. Here we describe the generation and characterization of two human hRSV-specific $CD4^+$ T cell clones (TCC) associated with type 0-like cytokine profiles. TCC 1 was specific for the matrix protein and restricted over HLA-DPB1*1601, while TCC 2 was specific for the attachment protein G and restricted over either HLA-DPB1*0401 or 0402. Interestingly, the latter epitope is conserved in both hRSV A and B viruses. Given the high allele frequencies of HLA-DPB1*0401 and 0402 worldwide, this epitope could be widely recognized and boosted by recurrent hRSV infections. Indeed, peptide stimulation of peripheral blood mononuclear cells from healthy adults resulted in detection of specific responses in 8 of 13 donors. Additional G-specific TCCs were generated from three of these cultures, which recognized the identical (*n*=2) or almost identical (*n*=1) HLA-DP4-restricted epitope as TCC 2. No significant differences were found in the capacity of cell lines obtained from infants with a severe (*n*=41) or mild (*n*=46) hRSV lower respiratory tract infection to function as antigen presenting cells to the G-specific TCC, suggesting that severity of hRSV disease is not linked to the allelic frequency of HLA-DP4. In conclusion, we have identified an hRSV G-specific human T helper cell epitope restricted by the widely expressed HLA class II alleles DPB1*0401 and 0402. Its putative role in protection and/or immunopathogenesis remains to be determined.

Introduction

Human respiratory syncytial virus (hRSV), a member of the genus *Pneumovirus* of the family *Paramyxoviridae*, is a major cause of severe lower respiratory disease in infants, immunocompromised individuals and the elderly [17,18]. hRSV infections cause yearly epidemics in the winter season of moderate climate zones and are most often associated with relatively mild upper respiratory tract infections [17]. In general, specific immunity is insufficient for protection and hRSV infections continue to occur throughout life.

At present, no licensed hRSV vaccine is available. During vaccine trials in the 1960s, vaccination with a formalin-inactivated whole virus preparation (FI-hRSV) was found to predispose for enhanced clinical disease following natural infection with hRSV [85]. Although the exact mechanism of this apparently immunopathological phenomenon remains unclear, studies in both rodent and non-human primate models have suggested that a skewed hRSV-specific T helper type 2 (Th2) response was a key factor in this process [86,128]. Several studies have suggested that primary infections in young infants, resulting in severe hRSV bronchiolitis, are also associated with Th2

responses [87,91]. However, we have studied hRSV-specific cellular immune responses in two cohort studies of infants with either severe hRSV bronchiolitis or relatively mild hRSV upper respiratory tract infection, and were unable to confirm this observation [88,225].

Few studies have described the hRSV-specific T cell response at the epitope level. In rodents four T cell epitopes have been described of which three were MHC class I- and one MHC class II-restricted. The MHC class I-restricted epitopes were located in the hRSV F [72,73] and M2 [74] proteins, whereas the MHC class II-restricted epitope was located in the hRSV G protein [66]. In humans, HLA class I-restricted epitopes have been identified in the hRSV F [67,226] and NP [68,69] proteins. However, no HLA class II-restricted T cell epitopes have been described. Recently, van Bleek *et al.* [70] described the human CD4 response to the hRSV F protein. Using a set of overlapping peptides they were able to demonstrate *ex vivo* F-specific CD4 memory T cell responses. Similar hRSV F-specific CD4 responses were also described earlier by Levely *et al.* [71].

In the present study, we describe two hRSV-specific $CD4^+$ T cell clones (TCC) generated from clinical materials collected from infants during the acute or convalescent phase of a laboratory-confirmed hRSV infection. Interferon-gamma (IFN-γ) ELISPOT assays were used to determine protein specificity, minimal epitope and HLA restriction element.

Materials & Methods

Antigen presenting cell lines

Autologous B-lymphoblastic cell lines (BLCL) were established by transformation of peripheral blood mononuclear cells (PBMC) with Epstein-Barr virus as described previously [198]. For the generation of target and/or stimulator cells, BLCL were infected with hRSV A2 (ATCC-VR1322) at a multiplicity of infection (moi) of 100, resulting in persistently infected BLCL (BLCL-hRSV) as described previously [199]. The percentage of hRSV-positive cells was checked by fluorescence activated cell scanner (FACScan) with FITC-labeled hRSV-specific monoclonal antibodies (mAbs; Imagen, DAKO, Glostrup, Denmark). All BLCL were maintained in RPMI1640 medium (BioWhittaker, Verviers, Belgium) containing penicillin (100 U/ml; BioWhittaker), streptomycin (100 µg/ml; BioWhittaker), L-glutamine (2mM; BioWhittaker), β-mercapto-ethanol (10^{-5}M; Merck KGaA, Darmstadt, Germany) [further referred to as culture medium, CM], supplemented with 10% heat-inactivated (HI; 30 min, 56°C) fetal bovine serum (FBS; Greiner, Frickenhausen, Germany). BLCL used for restriction element analysis were obtained from the European Collection of Cell Cultures (ECACC; cell lines represented by "I" followed by the IHW number), or were in-house generated (represented by "E" followed by an unique number). For autologous and in-house generated BLCL, molecular typing of the HLA-DRB1*, DQB1* and

DPB1* loci was performed using a commercial typing system (GenoVision, Vienna, Austria).

Generation of hRSV-specific T cell clones

TCC 1 was generated from nasal brush cells collected from an infant (age 20 months) during the acute phase of a laboratory-confirmed hRSV-mediated upper respiratory tract infection. Nasal brush T cells were stimulated *in vitro* with autologous γ-irradiated (3000 rad) BLCL-hRSV, and TCCs were generated by limiting dilution as described before [227]. In short, T cells were seeded in 60-well Terasaki plates (Greiner Bio-One, Frickenhausen, Germany) at concentrations of 3, 1 and 0.3 cells per well and stimulated with allogeneic feeder cells and recombinant human IL-2 (rhIL-2; Red Swan, Utrecht, The Netherlands). After two weeks of expansion, positive cultures were restimulated specifically using γ-irradiated autologous BLCL-hRSV, expanded with rhIL-2 and tested for hRSV-specificity in an IFN-γ ELISPOT assay (see below) after stimulation with BLCL or BLCL-hRSV. hRSV-specific TCCs were phenotyped by fluorescence using mAbs against CD3, CD4 and CD8 (DAKO).

TCC 2 was generated from PBMC collected from another infant (age 2 months) during the convalescent phase of an hRSV-mediated lower respiratory tract infection. PBMC were stimulated *in vitro* with γ-irradiated autologous BLCL-hRSV and TCCs were generated by limiting dilution as described above.

TCC P1, P2 and P3 were generated from PBMC collected from healthy adult donors. PBMC were stimulated *in vitro* with peptide $G_{158-189}$ (0.01μM) and TCC were generated by limiting dilution as described above.

T cells and T cell lines were maintained in CM supplemented with 10% HI human pooled serum (further referred to as R10H) and rhIL-2.

ELISPOT assay

Reactivity of TCC with antigen presenting cells (APC) was determined in IFN-γ ELISPOT assays as described previously [225]. In short, TCC were seeded in a concentration ranging from 4,000 to 10,000 cells per well in a 96-well V-bottom plate (Greiner Bio-One), APC were added in an effector-to-target ratio (E:T) of 1:5 and incubated for one hr at 37°C. Cells were transferred to nylon bottom plates (Nalge Nunc, Rochester, NY, USA) pre-coated with a mAb against IFN-γ (1-D1K; Mabtech AB, Stockholm, Sweden), incubated for four hr at 37°C, and subsequently washed with phosphate buffered saline containing 0.05% Tween-20 (Merck KGaA). Spots were visualized by incubation with a secondary biotinylated mAb against IFN-γ (7-B6-1; Mabtech AB), streptavidine alkaline phosphatase (Mabtech AB) and NBT-BCIP (Kirkegaard & Perry Laboratories, Gaithersburg, MA, USA). Finally, the color reaction was stopped by washing the plates with distilled water and spots were counted using a stereomicroscope at a 25-fold

magnification. IFN-γ ELISPOT results are shown in figures and tables as IFN-γ spot forming cells (SFC) per well.

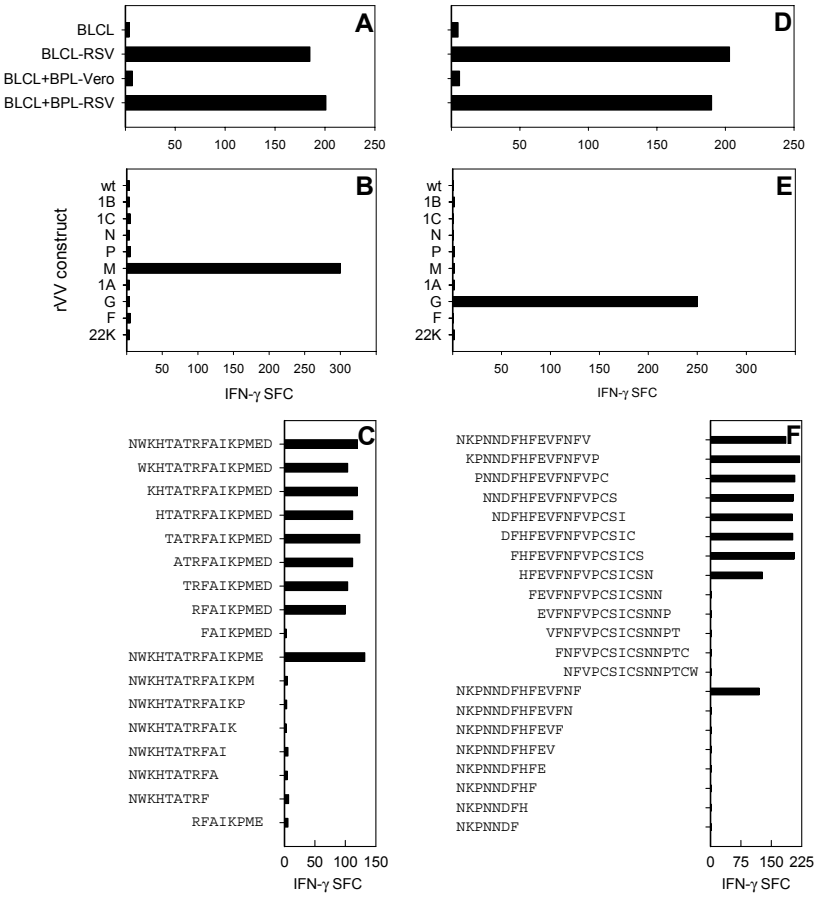

Figure 1: hRSV-specificity of TCC 1 (A) and TCC 2 (D) was determined in an IFN-γ ELISPOT, using autologous BLCL either or not infected with hRSV (BLCL-RSV and BLCL) or pulsed with antigen (BLCL + BPL-Vero and BLCL + BPL-RSV). Protein specificity of TCC 1 (B) and TCC 2 (E) was determined using autologous BLCL infected with different rVV constructs. The minimal epitopes recognized by TCC 1 (C) and TCC 2 (F) were determined using autologous BLCL pulsed overnight with 0.1 (C) or 0.01μM (F) peptide. Results are shown as spot forming cells (SFC) per well.

RSV protein specificity

Recombinant vaccinia virus (rVV) constructs mediating the expression of the individual hRSV proteins (rVV-F, -G, -M, -P, -N, -1A, -1B, -1C, -L and -22K) and a wild-type vaccinia virus (VV-wt) were used to infect autologous BLCL at an moi of 10. rVV-infected BLCL were used as APC in IFN-γ ELISPOT assays to determine protein specificity.

Peptide-specific T cell responses

For evaluation of the responses to different peptides, BLCL were pulsed overnight with peptides at different concentrations (1 to 0.001μM) and subsequently used as APC in IFN-γ ELISPOT assays.

For TCC 1, 15-mer peptides (n=49) with 5 amino acids overlap spanning the M protein were tested (last peptide 16 amino acids). For fine T cell epitope mapping, peptides were constructed with one or more deletions on either the N-terminal or C-terminal side. Peptides with free N- and C-termini were synthesized as described before [228], dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml and diluted to 100μM in RPMI1640 (BioWhittaker).

For TCC 2, a 101-mer peptide spanning the conserved region of the hRSV G protein (G2Na, amino acid 130-230, kindly provided by Dr. U.F. Power, Centre d'Immunologie Pierre-Fabre, Saint-Julien-en-Genevois, France) and smaller peptides spanning different regions of G2Na ($G_{170-187}$, $G_{187-223}$, $G_{187-198}$, $G_{174-189}$ and $G_{158-189}$) were tested. For fine epitope mapping overlapping 15-meres with 14 amino acids overlap or deletion mutants were used.

Table I: Determination of HLA-restriction elements of TCC 1 and 2

A: TCC 1

BLCL:	Aut.[a]	E-0002	I-9019	I-9023	I-9050	I-9095	I-9041	I-9063
DRB1*	0301	0102	0301	0301			1104	1302
DRB1*	0701	0701[b]			0701	0701		
DQB1*	0201	0303	0201	0201		0201	0301	0604
DQB1*	0202	0501			0202	030302		
DPB1*	1101	0201	0402	0101	020102	0401	1101	
DPB1*	1601	0401				1301	0402	1601
IFN-γ SFC[c]	168	3	2	0	2	1	2	188

B: TCC 2

BLCL:	Aut.	E-0004	E-0001	E-7366	I-9003	I-9105	I-9026	E-0003	I-9013
DRB1*	0102	0102	0301	1501	0101	11041	0402	1301	1501
DRB1*	0701	0302	0701	0701				1501	
DQB1*	0303	0402	0201	0202		0603	0302	0602	0602
DQB1*	0501	0501	0202	0602	0501			0603	
DPB1*	0201	0101	1101	0301	1301	0201		0402	0402
DPB1*	0401	0301	1601	1001			0401	0401	
IFN-γ SFC[d]	126	1	0	3	1	3	145	130	122

[a] Aut. = autologous BLCL

[b] HLA class II phenotype that BLCL have in common with the autologous BLCL are shaded

[c] IFN-γ spot forming cells (SFC) per well for TCC 1 were obtained by using BLCL pulsed with $M_{241-256}$

[d] IFN-γ SFC per well for TCC 2 were obtained by using BLCL pulsed with $G_{158-189}$

Protein preparations used to pulse BLCL overnight at a concentration of 1 μg/ml were: β-propiolactone (Sigma Aldrich, St. Louis, MO) inactivated hRSV (BPL-RSV, virus cultured in Vero cells) and Vero cell antigen (BPL-Vero), as described by de Swart *et al.* [128].

hRSV-specific T cell responses in PBMC or cord blood mononuclear cells

PBMC collected from healthy adults or cord blood mononuclear cells (CBMC) were stimulated with peptide $G_{158-189}$ (0.1 or 0.01μM) in R10H, and expanded in the presence of rhIL-2. After 2.5 weeks of culture, outgrowth of specific cells was analyzed in a CD69 expression assay as described before [229]. In short, residual CD69 molecules were enzymatically removed (0.1% chymotrypsin type II [Sigma Aldrich] in PBS, 10 minutes, 37°C), and subsequently cells were stimulated with autologous BLCL pulsed with peptide $G_{158-189}$ (positive) or $G_{174-189}$ (negative). After six hours cells were washed, incubated with αCD3-FITC (DAKO), αCD69-PE (BD Pharmingen), αCD8-PerCP (BD Pharmingen) and αCD4-APC (BD Pharmingen) and analyzed in a FACSCalibur (BD Biosciences BV, Alphen a/d Rijn, The Netherlands).

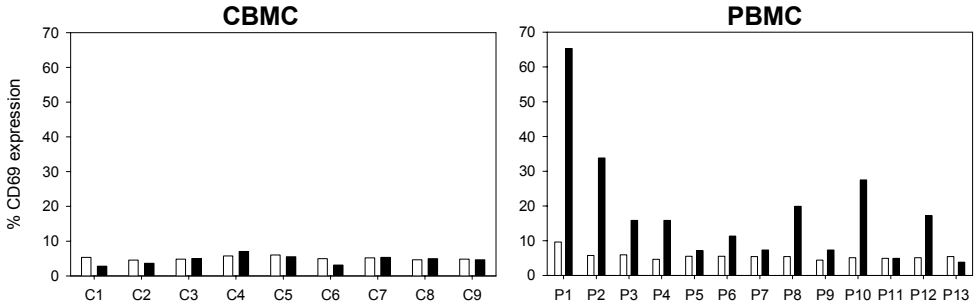

Figure 2: Detection of $G_{158-189}$-specific T cells in CBMC (C1-C9) or PBMC (P1-P13) stimulated for 2.5 weeks with 0.01μM peptide $G_{158-189}$. The percentage of $CD69^+$ cells in the $CD3^+CD4^+$ fraction of the expanded bulk cultures was determined after six hr of stimulation with autologous BLCL pulsed with $G_{174-189}$ (negative; open bars) or $G_{158-189}$ (positive; black bars). Responses were considered positive when the ratio of the percentage $CD69^+$ cells after stimulation with the positive and negative peptide was more than two.

Cytokine ELISAs

To determine cytokine profiles, TCCs (10^5 cells per well) were stimulated *in vitro* with autologous BLCL either or not infected with hRSV, or pulsed overnight with peptides (0.1μM) $M_{245-256}$ and $M_{241-251}$ (TCC 1 positive and negative peptide, respectively) or $G_{161-175}$ and $G_{167-181}$ (TCC P3 positive and negative peptide, respectively), at an E:T ratio of 2:1. After 5 days, culture supernatants were harvested and cytokine levels were determined according to the manufacturer's instructions for IFN-γ, IL-2, IL-4 and IL-5 (Biosource, Fleurus, Belgium) and IL-13 (U-Cytech, Utrecht, The Netherlands).

Table II: Determination of HLA-restriction elements of TCC P1, P2 and P3

A: TCC P1

BLCL:	Aut.[a]	I-9060	E-7366	I-9013	I-9105	I-9016	I-9002	I-9026
DRB1*	1301	1301[b]	0701		11041	1602	0102	0402
DRB1*	1501		1501	1501				
DQB1*	0602		0602	0602		0301	0501	0302
DQB1*	0603	0603	0202		0603			
DPB1*	0401	1901	0301		0201		0401	0401
DPB1*	0402		1001	0402		0402		
IFN-γ SFC[c]	200	0	1	140	10	126	190	180

B: TCC P2

BLCL:	Aut.	I-9032	I-9038	I-9016	I-9043	E-1519	I-9013	I-9002
DRB1*	0401	0401		1602	1101	0801		0102
DRB1*	1201		1201			1401	1501	
DQB1*	0301	0302	0301	0301	0301	0402	0602	0501
DQB1*						0503		
DPB1*	0301	02012	02012		1001	0301		0401
DPB1*	0402			0402		0501	0402	
IFN-γ SFC	180	3	2	165	0	6	175	185

C: TCC P3

BLCL:	Aut.	I-9038	E-7366	I-9016	I-9043	E-0005	I-9105	I-9050	I-9002	I-9013
DRB1*	1201	1201	0701	1602	1101	1502	11041	0701	0102	
DRB1*	1501		1501							1501
DQB1*	0301	0301	0602	0301	0301			0202	0501	0602
DQB1*	0601		0202			0601	0603			
DPB1*	0201	02012	0301	0402	1001	0901	0201	02012		0402
DPB1*	0401		1001						0401	
IFN-γ SFC[=]	125	80	1	104	2	1	130	80	124	112

[a]Aut. = autologous BLCL
[b]HLA class II phenotype that BLCL have in common with the autologous BLCL are shaded
[c]IFN-γ spot forming cells (SFC) per well for TCC P1, P2 and P3 were obtained by using BLCL pulsed with $G_{158-189}$

Results

Generation and characterization of human hRSV-specific T cell clones

TCC 1 was cloned from nasal brush cells collected from an infant during the acute phase of a laboratory-confirmed hRSV upper respiratory tract infection. It was hRSV-specific (figure 1A), of the $CD3^+CD4^+$ phenotype and recognized the hRSV M protein (figure 1B). Of the overlapping peptides tested, peptide N_{241}WKHTATRFAIKPMED$_{256}$ was recognized, and the minimal epitope was R_{248}FAIKPME$_{255}$ with an additional amino acid on either side (figure 1C).

TCC 2 was cloned from PBMC collected from an infant during the convalescent phase of an hRSV lower respiratory tract infection. It was hRSV-specific (figure 1D), of the $CD3^+CD4^+$ phenotype and recognized the hRSV G protein (figure 1E). The clone was also found to recognize BLCL pulsed with G2Na ($G_{130-230}$) or with $G_{158-189}$ (data not shown). The minimal epitope was F_{163}HFEVFNFV$_{171}$ (figure 1F).

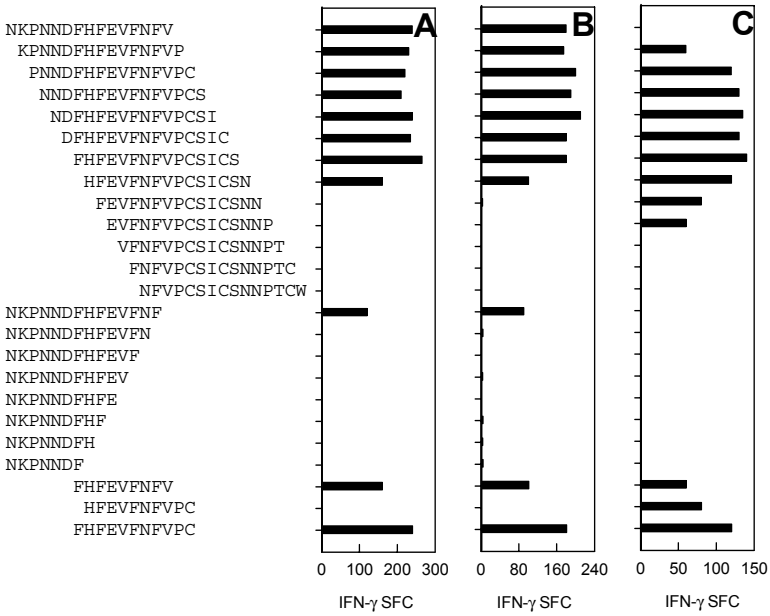

Figure 3: Determination of the minimal epitopes of TCC P1, P2 and P3 in an IFN-γ ELISPOT assay using autologous BLCL pulsed with 0.01μM $G_{158-189}$ as APC. Results are shown as spot forming cells (SFC) per well.

Analysis of published sequences suggested that the M epitope is conserved in hRSV A but not in hRSV B viruses, whereas the G epitope is conserved in both subgroups (results not shown). This was confirmed in an IFN-γ ELISPOT assay using autologous BLCL pulsed with hRSV A (L strain, ATCC-VR26) and hRSV B (B1 strain, ATCC-1400) antigen as APC. Where the G-specific TCC P2 was able to recognize both hRSV A and B pulsed APC, the M-specific TCC 1 was only capable of recognizing hRSV A pulsed APC (data not shown).

Determination of HLA-restriction elements of TCC 1 and 2

The class II genotype of the donor from whom TCC 1 originated was HLA-DRB1*0301,0701; DQB1*0201,0202; DPB1*1101,1601; for the donor of TCC 2 this was HLA-DRB1*0102,0701; DQB1*0303,0501; DPB1*0201,0401. By testing reactivity with peptide-pulsed BLCL matched or mismatched for HLA-DR or -DQ we were unable to determine the

restriction pattern for either of these TCC: for TCC 1 no responses were found, while for TCC 2 the majority of the APCs resulted in positive responses. When the HLA-DP alleles were included, it was found that TCC 1 was restricted by HLA-DPB1*1601 (table IA), while TCC 2 recognized peptide in the context of either HLA-DPB1*0401 or 0402 (table IB).

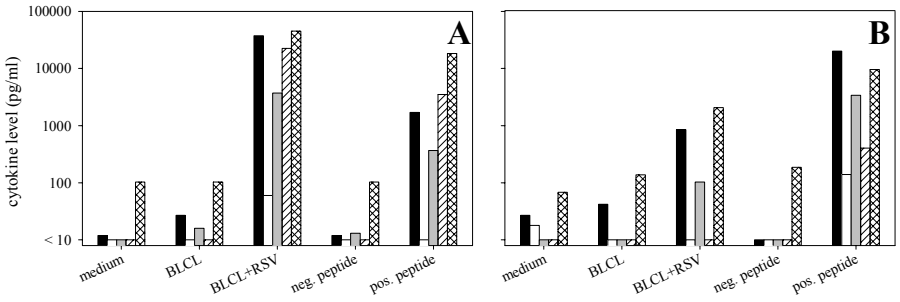

Figure 4: Cytokine levels in culture supernatants of TCC 1 (A) or TCC P3 (B) five days after stimulation with medium, autologous BLCL (BLCL), autologous BLCL-hRSV (BLCL-RSV), autologous BLCL pulsed with negative peptide ($M_{241-251}$ for TCC 1 and $G_{167-181}$ for TCC P3) or autologous BLCL pulsed with positive peptide ($M_{245-256}$ for TCC 1 and $G_{161-175}$ for TCC P3). Detection limits were 10 pg/ml for IFN-γ (black bars), IL-2 (white bars), IL-4 (gray bars) and IL-5 (hatched bars) and 100 pg/ml for IL-13 (double-hatched bars).

Peptide $G_{158-189}$ responses in the human population

Since HLA-DPB1*0401 and 0402 are the most frequent HLA class II alleles in the human population [230], we investigated the response to peptide $G_{158-189}$ in PBMC obtained from healthy adult donors and in CBMC. No specific T cells were found in the CBMC cultures, while $G_{158-189}$-specific T cells were detected in 8 of 13 PBMC cultures (figure 2).

In order to confirm that the $G_{158-189}$-expanded cells were indeed hRSV-specific T cells, TCC were generated from three of these cultures (P1, P2 and P3), and the minimal epitopes of these TCC were determined. As shown in figure 3, TCC P1 and P2 recognized the same minimal epitope as TCC 2, while TCC P3 recognized a 10-mer peptide shifted one amino acid in the C-terminal direction, H_{164}FEVFNFVPC_{173}.

The class II genotype of donor P1 was HLA-DRB1*1301,1501; DQB1*0602,0603; DPB1*0401,0402, of donor P2 HLA-DRB1*0401,1201; DQB1*0301; DPB1*0402,0301, and of donor P3 HLA-DRB1*1201,1501; DQB1*0301,0601; DPB1*0401,0201. Similar to TCC 2, both TCC P1 and P2 recognized peptide in the context of HLA-DPB1*0401 or 0402 (table IIA and IIB). Interestingly, TCC P3 was also able to recognize peptide in the context of HLA-DPB1*0201, and 02012 (table IIC).

All TCC produce both type 1 and 2 cytokines

TCC 1 (two subclones), P2 and P3 were tested for their ability to produce cytokines after stimulation with different antigens. All TCCs showed

specific cytokine production after stimulation with hRSV-infected or peptide-pulsed autologous BLCL, predominated by IFN-γ and IL-13. Interestingly, while TCC 1 (both subclones) produced higher levels of IL-5 than IL-4 upon stimulation, TCCs P2 and P3 produced more IL-4 than IL-5 (figure 4 and data not shown).

HLA-DP4 phenotype in infants with hRSV infections of different clinical severity

To determine if the T cell response to the HLA-DP4-restricted G epitope plays a role in the pathogenesis of severe hRSV disease, the allelic frequency of HLA-DP4 or the precursor frequency of the G-specific T cells could be compared between infants with hRSV disease of different severity. As alternative to the determination of HLA-DP4 allelic frequencies, specimens from a previous cohort study [88] allowed us to test the functional capacity of BLCL obtained from infants with a severe or mild hRSV lower respiratory tract infection to function as APC to the G-specific TCC. Positive responses of the TCC were found after stimulation by 35/41 (85%) and 50/64 (78%) peptide-pulsed BLCL, respectively (no significant difference, $p>0.1$, Fisher's Exact Test, 2-sided).

Discussion

We have identified two HLA-DP-restricted T helper cell epitopes in the hRSV M and G protein, conserved in subgroup A or in both subgroup A and B, respectively. The M epitope was recognized by a TCC restricted over HLA-DPB1*1601, while the G epitope was recognized in the context of either HLA-DPB1*0401 or 0402. The ubiquitous distribution of the latter alleles and the results of our peptide PBMC bulk stimulations suggest that responses to the G epitope are generated in a large part of the human population.

The majority of studies on human T helper cell responses have focused on HLA-DR- and -DQ-restricted T cells, to a large extent because HLA-DP appeared less important in contributing to the risk of graft-versus-host disease (GVHD) [231]. However, several HLA-DP-restricted T cell epitopes have now been described [232], including viral epitopes [233,234]. To our knowledge, the hRSV M-specific TCC described here identifies the first HLA-DPB1*1601-restricted epitope. Recently, a number of TCC have been described recognizing their epitope in the context of HLA-DPB1*0401 and/or 0402, which both belong to the serologically defined HLA-DPw4 antigenic group [235]. HLA-DPw4 is the most prevalent HLA class II antigen, with an allelic frequency of 78% in the Caucasian population [236]. The hRSV G epitope identified in the present paper is consistent with the suggested motif for HLA-DP4-restricted epitopes as described previously [230].

HLA-DPB1*0401 and 0402 differ by only three amino acids, and TCC raised in subjects with HLA-DPB1*0401 were in some cases also able to recognize their antigen in the context of HLA-DPB1*0402 [235]. We have found the same in the donor of TCC 2 and in donor P3, but have also raised a TCC in a donor who was HLA-DPB1*0402 positive that could recognize its epitope in the context of HLA-DPB1*0401 (donor P3). This last TCC, which recognized an epitope, which was shifted one amino acid in the C-terminal direction as compared to TCCs 2, P1 and P2, could also recognize its epitope in the context of HLA-DPB1*0201 and 02012. Interestingly, HLA-DPB1*0201 and HLA-DPB1*0402 differ by only one amino acid in the P4 pocket of the peptide-binding groove, and HLA-DP2 and -DP4 were previously suggested to form a supertype of class II molecules on basis of the homology in the peptide-binding pockets P1 and P6 [230].

The functional studies using BLCL from infants with mild or severe hRSV-mediated lower respiratory tract disease as APC to the G-specific TCC suggested that severity of hRSV disease is not linked to the allelic frequency of HLA-DP4. However, frequency studies of epitope-specific T cells in infants with different disease severity will have to be performed to determine whether these play a role in hRSV pathogenesis. The fact that the conserved HLA-DP4-restricted epitope was found in the G protein brings another dimension to this question, since G-specific responses have often been suggested to be involved in natural or vaccine-mediated enhanced disease [64,191]. However, in our study similar Th0-like cytokine production profiles were found for both the M- and G-specific TCCs.

Castelli *et al.* [230] suggested that specific HLA-DP4-restricted epitopes could be used as peptide vaccines, because of the high frequency of HLA-DP4 worldwide. However, vaccination with non-replicating hRSV vaccines that induce only HLA class II-restricted T cell responses has been associated with immunopathology in humans [85], non-human primates [128,237] or rodents [86,172].

In conclusion, we have identified the first HLA-DPB1*1601-restricted and a conserved HLA-DP4-restricted T cell epitope in the hRSV M and G protein, respectively. Whether immune responses to the latter epitope are involved in hRSV-mediated immunopathogenesis remains to be determined.

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Chapter 3.3

T cell responses to the respiratory syncytial virus fusion and attachment proteins in recombinant vaccinia virus MVA stimulated human bulk cultures.

L. de Waal, Y. Süzer, L.S. Wyatt, K. Sintnicolaas, G. Sutter,
B. Moss, A.D.M.E. Osterhaus, R.L. de Swart

submitted

Abstract

We have studied memory T cell responses to the hRSV fusion (F) and attachment (G) proteins in peripheral blood mononuclear cells (PBMC) from healthy young adults. Stimulation with autologous cells infected with recombinant modified vaccinia virus Ankara expressing F (rMVA-F) yielded F-specific $CD4^+$ and $CD8^+$ T cell lines, while rMVA-G-stimulated PBMC only yielded G-specific $CD4^+$ T cell responses. Using a set of overlapping peptides spanning the F protein, previously undescribed epitope-containing regions were defined. This approach will be useful to define protein-specific T cell responses in different viral systems.

Introduction

Infection with human respiratory syncytial virus (hRSV), a member of the genus *Pneumovirus* of the family *Paramyxoviridae*, is a major cause of severe respiratory disease in infants, immunocompromised individuals and the elderly. hRSV infections cause yearly epidemics in the winter season of moderate climate zones and are commonly associated with relatively mild upper respiratory tract infections [17]. Specific immunity induced by natural infection generally does not confer protection from re-infection, but does protect against the development of severe lower respiratory tract disease. No human hRSV vaccine is licensed to date, but a number of different vaccination approaches are currently being explored in preclinical and clinical studies [118]. However, vaccine development is still hampered by the results of clinical trials in the 1960s, when formalin-inactivated whole virus preparations were found to predispose for enhanced disease upon natural infection with hRSV [85]. Although the exact mechanism remains subject of debate, it is generally accepted that this enhanced disease had an immunopathological basis [86,129,172].

It has often been speculated that the hRSV G protein plays a role in the immunopathogenesis of vaccine-mediated or naturally occurring severe hRSV disease [64]. The G protein has several unique properties, including heavy glycosylation, the occurrence of mucin-like regions and the apparent inability to induce an MHC class I-restricted $CD8^+$ cytotoxic T lymphocyte (CTL) response [64]. Studies characterizing the hRSV-specific cellular immune responses in humans remain limited. HLA class I- and/or class II-restricted T cell epitopes have been described in the nucleoprotein (N), fusion protein (F) and attachment protein (G). HLA class II-restricted T cell responses to the F protein were studied in relative detail by using a set of overlapping peptides, which resulted in the detection of multiple epitope-containing regions spanning the F protein [70,71].

The precursor frequencies of hRSV-specific human T cells present in peripheral blood have not been studied. However, to detect low frequent memory T cells a specific *in vitro* stimulation protocol is required. In contrast to the approach using *in vitro* stimulation with hRSV-infected cells [226] or

overlapping peptides [70], we have chosen to use recombinant modified vaccinia virus Ankara (rMVA) expressing the hRSV F (rMVA-F) or G (rMVA-G) genes to enrich for hRSV glycoprotein-specific T cells. MVA is replication-deficient in most mammalian cells, and was shown to be a highly effective and safe vaccine vector system, capable of inducing both MHC class I- and class II-restricted T cells [163,167]. The purpose of the present study was the further characterization of the epitope recognition in the F- and G-specific T cell response against hRSV, which may be of crucial importance for the rational design of safe and effective hRSV vaccines.

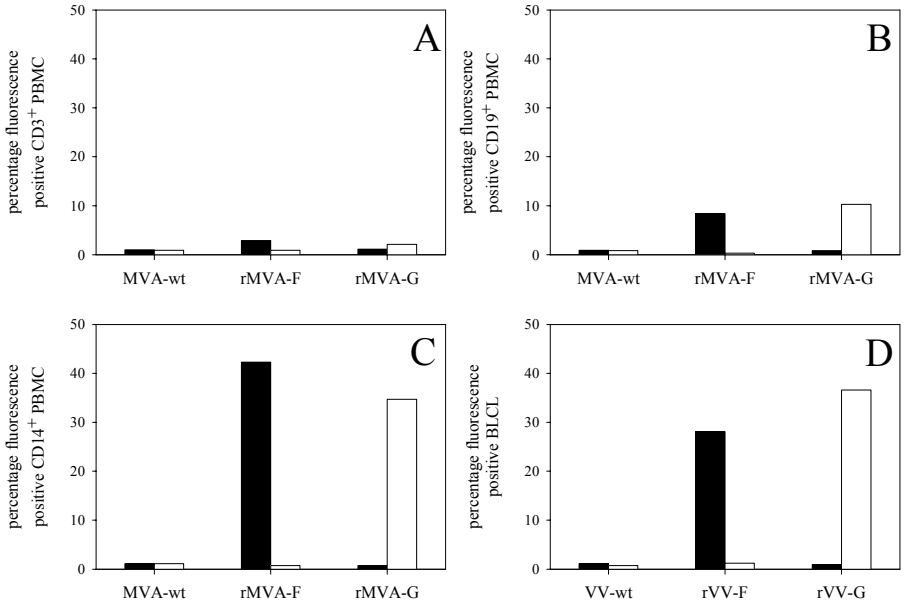

Figure 1: Detection of hRSV F or G in $CD3^+$ PBMC (A), $CD19^+$ PBMC (B), $CD14^+$ PBMC (C) or BLCL (D). PBMC were infected with MVA-wt, rMVA-F or rMVA-G, while BLCL were infected with VV-wt, rVV-F or rVV-G. Cells were stained using monoclonal antibodies to F (black bar) or G (white bar).

Subjects, Materials & Methods

Peripheral blood mononuclear cells (PBMC) were collected from three healthy young adult blood donors with immunity to hRSV and without immunity to vaccinia virus (VV). The HLA class I and class II genotype of these donors was for donor 1: HLA-A*11; B*35,44; Cw*04; DRB1*11,13; DQB1*03,06; DPB1*0201,1301; donor 2: HLA-A*01,03; B*07,35; Cw*04,07; DRB1*12,14; DQB1*03,05; DPB1*0401,0402; and donor 3: HLA-A*01,03; B*08,15; Cw*03,07; DRB1*01,11; DQB1*03,05; DPB1*0201,1401.

PBMC of the first and second donor (10^5 cells per well, 96-well round bottom plates) were stimulated with autologous monocytes (approximately 10^4 per well) infected (multiplicity of infection [moi] of 3) with rMVA-F or rMVA-G [156] or with wild-type MVA (MVA-wt). MVA viruses were grown in chicken embryo fibroblasts, purified by ultracentrifugation through sucrose and reconstituted in physiological saline. PBMC were cultured in RPMI1640 (BioWhittaker) supplemented with heat-inactivated human pooled serum, after 3 days recombinant human interleukin-2 (rhIL-2, Red Swan) was added and expansion of T cells was observed 3-5 days later in the F- and G-stimulated cultures. No expansion was found in the MVA-wt-stimulated cultures. After 2-4 weeks, individual wells were re-stimulated with autologous monocytes infected with the homologous rMVA construct and expanded with rhIL-2. In some cases these cultures were further expanded by stimulation with phytohaemagglutinin (Roche) and γ-irradiated (3000 rad) allogeneic feeder cells.

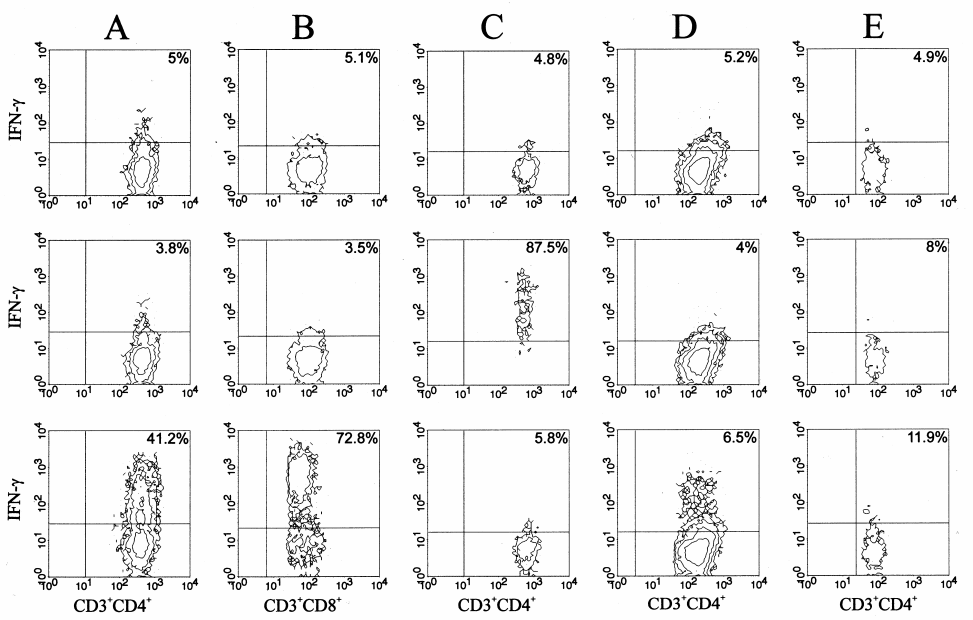

Figure 2: F-specific (A, B and D), G-specific (C) or false-positive (E) T cell responses in bulk cultures. Bulk cultures were stimulated in a six hr assay with autologous BLCL infected with VV-wt (top graphs), rVV-G (middle graphs) or rVV-F (bottom graphs) and subsequently stained for CD3, CD4, CD8 and IFN-γ.

PBMC of the third donor (5 x 10^4 cells per well) were stimulated twice with γ-irradiated autologous rMVA infected (moi 3) PBMC (1.5 x 10^5 cells per well) and expanded using rhIL-2. Infection of PBMC resulted in surface expression of F or G in $CD14^+$ (40% and 34%, respectively; figure 1C),

$CD19^+$ (8% and 10%, respectively; figure 1B) and $CD3^+$ (2% and 1%, respectively; figure 1A) cells as determined by FACS analysis.

For evaluation of specificity, autologous Epstein-Barr virus-transformed B lymphoblastic cell lines (BLCL) were infected with hRSV A2 (ATCC-VR1322) or recombinant VV (rVV) expressing F or G, or pulsed with peptides at a concentration of 1μM. These included overlapping peptides spanning the hRSV A2 F protein (NCBI accession number AAB86664; 18 amino acids in size with 12 overlap [226]) and five different hRSV A2 G peptides (NCBI accession number AAB86663; $G_{130-230}$, $G_{158-189}$, $G_{174-189}$, $G_{169-183}$ or $G_{162-175}$). Staining of rVV-F- or rVV-G-infected BLCL with F- and G-specific monoclonal antibodies showed a surface expression in 27% and 36% of the cells, respectively (figure 1D).

Reactivity of specific bulk cultures with antigen-presenting cells (APC) was determined in interferon-gamma (IFN-γ) ELISPOT, intracellular IFN-γ FACS staining or induction of FACS-measured CD69 expression. IFN-γ ELISPOT assays (Mabtech AB) were performed as described [225], and results were considered positive if the number of spots was more than three times the background. Intracellular IFN-γ FACS staining was performed after six hr restimulation of bulk cultures in the presence of GolgiStop (Pharmingen), followed by surface staining with anti CD3, CD4 and CD8 and intracellular staining with anti IFN-γ. CD69 induction assays were performed as described [229]: after enzymatic (0.1% chymotrypsin type II [Sigma Aldrich]) removal of residual CD69 molecules, cultures were re-stimulated and surface expression of CD3, CD4, CD8 and CD69 was analyzed by FACSCalibur (Becton Dickinson). For analysis, T cells were gated on basis of their FSC/SSC scatter and CD3, CD4 and CD8 expression. $CD4^+$ or $CD8^+$ T cell populations were analyzed if they consisted of more than 2% of the total $CD3^+$ population. For each bulk culture quadrants were set in the $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell populations stimulated with uninfected or wild-type VV (VV-wt)-infected autologous BLCL at a level resulting in approximately 5% CD69 or IFN-γ expressing cells.

Results

rMVA-F- and -G-stimulated bulk cultures were first screened for the presence of hRSV-specific T cells in IFN-γ ELISPOT and/or CD69 induction assay. Bulk cultures positive in either of these two assays were further analyzed in an intracellular IFN-γ fluorescence assay using autologous BLCL infected with VV-wt, rVV-F or rVV-G as APC. In bulk cultures stimulated with rMVA-F, F-specific T cells could be detected of both the $CD4^+$ and $CD8^+$ phenotype, of which examples are shown in figure 2A and B. In bulk cultures stimulated with rMVA-G, G-specific $CD4^+$ T cells could be detected, of which an example is shown in figure 2C. The responses of all bulk cultures are shown in figure 3. Although some bulk cultures contained relatively high percentages of specific T cells similar to the examples shown in figure 2A-C, responses detected in other cultures were only slightly higher

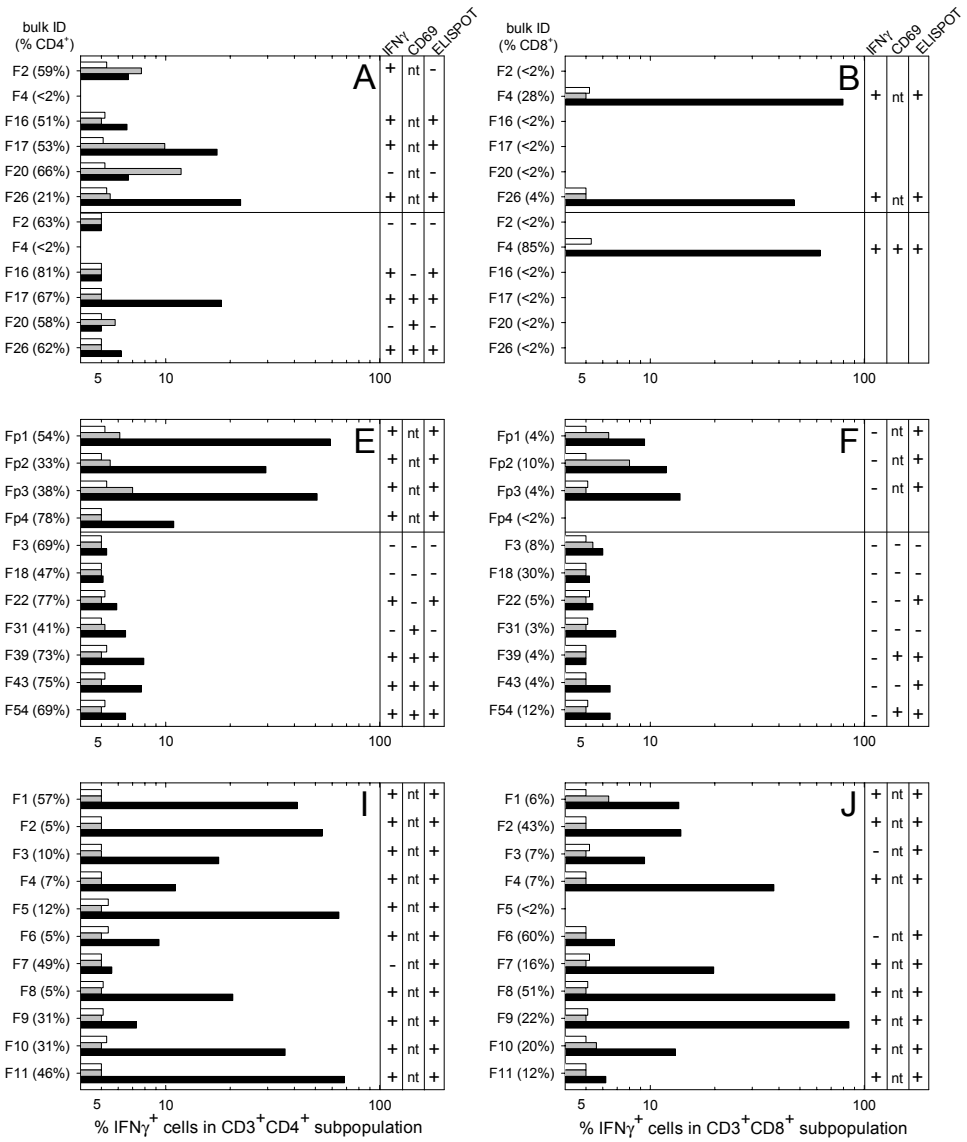

Figure 3[1]: Overview of F-specific T cell responses in single (F*n*) or pooled (Fp*n*) bulk cultures of donor 1 (A-B), 2 (E-F) or 3 (I-J). Bulk cultures had been expanded with rMVA-F and percentages of IFN-γ positive cells were determined after a six hour stimulation with autologous BLCL infected with VV-wt (white bars), rVV-G (grey bars) or rVV-F (black bars) in the $CD3^+CD4^+$ (A, E, I) or $CD3^+CD8^+$ (B, F, J) population. Quadrants were set in the $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell populations stimulated with VV-wt infected autologous BLCL at a level resulting in approximately 5% IFN-γ expressing cells. The ID of the bulk culture is followed by the percentage of $CD4^+$ or $CD8^+$ cells in the total $CD3^+$ population (between brackets). The qualitative results of the intra-cellular IFN-γ staining, CD69 assay and IFN-γ ELISPOT assay are shown in columns on the right of each figure panel. nt = not tested.

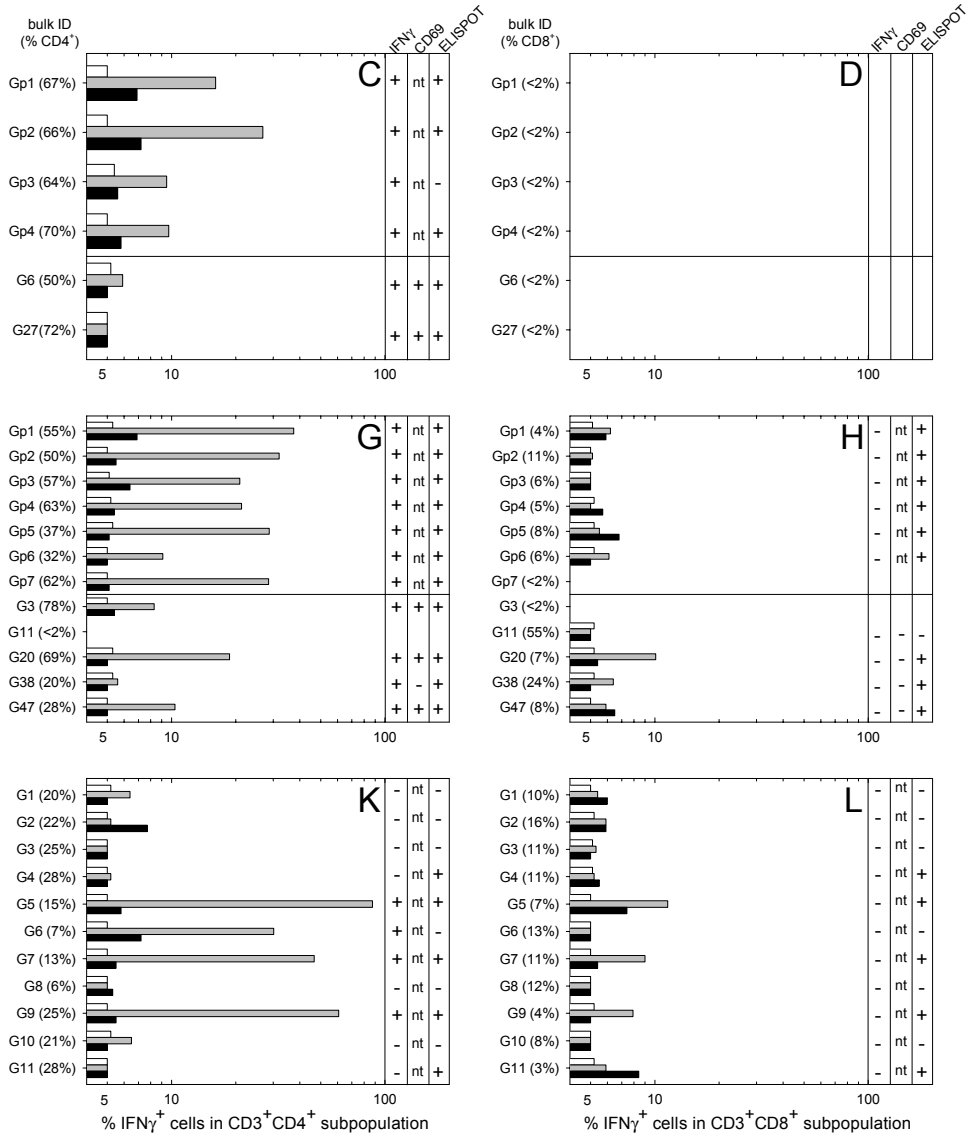

Figure 3[2]: Overview of G-specific T cell responses in single (G*n*) or pooled (Gp*n*) bulk cultures of donor 1 (C-D), 2 (G-H) or 3 (K-L). Bulk cultures had been expanded with rMVA-G and percentages of IFN-γ positive cells were determined after a six hour stimulation with autologous BLCL infected with VV-wt (white bars), rVV-G (grey bars) or rVV-F (black bars) in the $CD3^+CD4^+$ (C, G, K) or $CD3^+CD8^+$ (D, H, L) population. Quadrants were set in the $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell populations stimulated with VV-wt infected autologous BLCL at a level resulting in approximately 5% IFN-γ expressing cells. The ID of the bulk culture is followed by the percentage of $CD4^+$ or $CD8^+$ cells in the total $CD3^+$ population (between brackets). The qualitative results of the intra-cellular IFN-γ staining, CD69 assay and IFN-γ ELISPOT assay are shown in columns on the right of each figure panel. nt = not tested.

than the background. Of the latter cultures, some were clearly positive (see example in figure 2D), while others seemed to be false-positives with increased background (see example in figure 2E). The low positive G-specific $CD8^+$ T cell responses (donor 2, bulk culture G20; donor 3, bulk cultures G5, G7 and G9) all belonged to this last category. For each bulk culture tested the qualitative results of the intracellular IFN-γ staining, CD69 assay and IFN-γ ELISPOT assay are shown in columns on the right of each figure panel.

Selected specific rMVA-F-stimulated bulk cultures were tested in IFN-γ ELISPOT assays using autologous BLCL infected with hRSV or pulsed with overlapping peptides of the F protein. As shown in table I, separate bulk cultures of each donor were found to recognize different peptides. The positive peptides were subsequently tested in an intracellular IFN-γ assay using peptide-pulsed autologous BLCL as APC to determine the phenotype of the specific T cells.

Table I: F-specific responses in IFN-γ ELISPOT and IFN-γ fluorescence

		IFN-γ ELISPOT		IFN-γ fluorescence	
Donor	Bulk ID	BLCL-RSV	F peptide (n=94)	CD4	CD8
1	F4[a]	+	$F_{109-126}$		?[b]
	F16	+	$F_{139-156}$	$F_{139-156}$	
2	Fp1[c]	+	$F_{331-348}$, $F_{337-354}$	$F_{331-348}$, $F_{337-354}$	
	Fp2	+	$F_{295-312}$, $F_{337-354}$, $F_{463-480}$	$F_{295-312}$, $F_{337-354}$, $F_{463-480}$	
	Fp3	+	$F_{265-282}$, $F_{271-288}$	$F_{265-282}$, $F_{271-288}$	
3	F1	+	$F_{337-354}$	$F_{337-354}$	
	F2	+	$F_{133-150}$, $F_{139-156}$, $F_{229-246}$, $F_{235-252}$	$F_{133-150}$, $F_{139-156}$, $F_{229-246}$, $F_{235-252}$	
	F3	+	$F_{139-156}$, $F_{145-162}$, $F_{223-240}$, $F_{295-312}$, $F_{301-318}$, $F_{337-354}$	$F_{139-156}$, $F_{145-162}$, $F_{223-240}$, $F_{295-312}$, $F_{301-318}$, $F_{337-354}$	
	F4	+	$F_{139-156}$, $F_{289-306}$, $F_{295-312}$	$F_{139-156}$	$F_{289-306}$
	F5	+	$F_{433-450}$, $F_{439-456}$	$F_{439-456}$	
	F6	+	$F_{229-246}$, $F_{235-252}$	$F_{229-246}$, $F_{235-252}$	
	F7	+	$F_{169-186}$, $F_{235-252}$	$F_{169-186}$, $F_{235-252}$	
	F8	+	$F_{271-288}$, $F_{337-354}$, $F_{343-360}$	$F_{337-354}$	$F_{271-288}$
	F9	+	$F_{223-240}$, $F_{229-246}$, $F_{235-252}$, $F_{271-288}$	$F_{229-246}$	$F_{271-288}$
	F10	+	F_{25-42}, F_{31-48}, $F_{391-408}$, $F_{463-480}$	F_{25-42}, F_{31-48}, $F_{391-408}$, $F_{463-480}$	
	F11	+	$F_{163-180}$, $F_{169-186}$, $F_{241-258}$, $F_{247-264}$, $F_{391-408}$, $F_{463-480}$	$F_{163-180}$, $F_{169-186}$, $F_{241-258}$, $F_{247-264}$, $F_{391-408}$, $F_{463-480}$	

[a] F*n* = bulk culture originating from one well after stimulation with rMVA-F
[c] not confirmed in IFN-γ fluorescence, but bulk contained almost exclusively $CD8^+$ T cells
[b] Fp*n* = pool of several bulk cultures after stimulation with rMVA-F

Selected specific rMVA-G-stimulated bulk cultures were tested in IFN-γ ELISPOT assays using autologous BLCL infected with hRSV or pulsed with $G_{130-230}$, $G_{158-189}$, $G_{187-223}$, $G_{169-183}$ or $G_{162-175}$. The last peptide comprised a recently identified HLA-DP4-restricted T cell epitope [256]. The G-specific T cell lines tested (*n*=6) from donor 2, who was of the HLA-DPB1*0401 and 0402 genotype, were all specific for this peptide. The G-specific T cell lines of donors 1 and 3 mostly responded to the central conserved domain of the G protein ($G_{130-230}$; 2 of 4 and 3 of 3 lines tested, respectively).

Discussion

We generated hRSV F- and G-specific T cell lines by stimulation of human PBMC with rMVA-F- or rMVA-G-infected autologous cells. In previous studies with a similar objective, we had used rVV constructs for this purpose. Although this initially resulted in seemingly specific expansion of T cells, these cultures eventually all succumbed to VV infection. The replication deficiency of MVA ensures that cultures generated with the stimulation protocol used here stay free from infectious VV. An attractive property of using rMVA is that infected APC present peptides derived from the recombinant gene in the context of both HLA class I and class II.

In a recent study focusing on the characterization of the hRSV F-specific HLA class II-restricted T cell response, bulk cultures were generated after stimulation with pools of six consecutive overlapping peptides [70]. Comparison of the peptides recognized by specific T cells in both studies showed that new Th epitope-containing regions were identified in the present study: $F_{133-168}$, $F_{163-180}$, $F_{247-264}$ and $F_{433-456}$. Combination of the results of both studies clearly shows that Th epitope containing regions are scattered over the entire F protein.

A clear difference between both studies is that we were able to detect F-specific $CD8^+$ T cells, while the responses upon bulk stimulation with peptides were mediated by $CD4^+$ T cells only [70]. It is interesting to note that peptides that did not result in expansion of F-specific $CD8^+$ T cells after stimulation of PBMC with relatively high (20μM) peptide concentrations, could successfully be used for detection of F-specific $CD8^+$ T cells in our rMVA-F expanded bulk cultures after stimulation with peptide-pulsed autologous BLCL.

One of the additional objectives of the present study was an attempt to identify HLA class I-restricted G-specific CTL responses. However, whereas stimulation with rMVA-F resulted in the detection of F-specific CTL responses in two out of three donors, upon rMVA-G stimulation only G-specific Th cells could be demonstrated. Although in some cultures low G-specific CTL responses were detected, these all proved to be false positives, since the results could not be reproduced and re-stimulation with rMVA-G infected APC did not result in outgrowth of specific CTL. Since only three donors were used in the present study, it cannot be concluded that HLA class

I-restricted G-specific CTL do not exist. We therefore intend to extent the current approach to a larger number of individuals to address this issue, after optimization of the restimulation protocol using rMVA-F infected PBMC, monocytes or dendritic cells.

In almost all cases G-specific $CD4^+$ T cell responses were directed to the central conserved region of the G protein. However, this does not exclude that the variable regions of the G protein induce Th cell responses. Due to the high variability in the G protein, the G sequences of the viruses that infected the adult donors during their lifetime were probably relatively distant from that of the A2 virus used to generate the rMVA-G virus. Therefore, if Th responses to variable regions of the G protein existed in these donors, they might simply not have been stimulated by the rMVA-G virus.

In conclusion, we have identified in three healthy young adults HLA class I- and class II-restricted T cell responses to multiple regions of the F protein, while T cell responses to the G protein were all restricted over HLA class II and mainly directed to the central conserved domain of the G protein. This study shows that stimulation of PBMC with a recombinant viral vector mediating the expression of a single protein and the subsequent use of overlapping peptides can result in the identification of multiple candidate epitopes in a single donor. This approach will allow the study of protein-specific T cell responses in several viral systems.

Acknowledgements

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Chapter 4.1

Evaluation of BBG2Na in infant macaques: specific immune responses after vaccination and RSV challenge.

L. de Waal, U.F. Power, S. Yüksel, G. van Amerongen, T.N. Nguyen, H.G.M. Niesters, R.L. de Swart, A.D.M.E. Osterhaus

Vaccine (*in press*)

Abstract

We have addressed the safety of alum-adsorbed BBG2Na, a recombinant hRSV subunit vaccine, in infant macaques. Animals received two vaccinations, and were challenged four months later with hRSV. In two of four BBG2Na-vaccinated animals, specific IL-13 producing T cells were detected. Upon challenge low level pulmonary eosinophilia was observed in the same two animals. Although the levels of these responses were substantially lower than those observed in the FI-hRSV controls, these data suggest that more extensive studies focusing on immunopathological safety of alum-adsorbed BBG2Na in non-human primates would be required before proceeding to clinical trials in seronegative infants.

Introduction

Human respiratory syncytial virus (hRSV) is a major cause of severe respiratory tract disease in infants and the elderly [17]. Despite its clinical relevance, no licensed hRSV vaccine is available. Several different vaccine approaches have been explored in pre-clinical studies, and a number of candidate vaccines are presently being evaluated in human clinical trials [118]. However, the potential use of non-replicating hRSV vaccines in seronegative infants is still being held back by observations in the 1960s, when experimental FI-hRSV preparations formulated in alum were found to predispose for severe disease upon subsequent natural hRSV infection [85]. During the same period, vaccine-mediated enhanced disease was also observed after vaccination with a similar formalin-inactivated whole virus preparation of another member of the family *Paramyxoviridae*, namely measles virus [238]. Since the mechanisms of these apparently immunopathological phenomena are still debated [64,86,128,129,172], it remains unclear which specific information would be required for a new generation non-replicating hRSV vaccine to proceed to clinical trials in seronegative infants.

A large number of hRSV vaccination and challenge studies have been carried out in mice and cotton rats. Since correlates of FI-hRSV-mediated immunopathology have been or are being established in these species, they allow comparison of the immunopathological safety of candidate new vaccines with FI-hRSV. However, it remains difficult to extrapolate results obtained in SPF inbred rodents to humans. As a final step in the pre-clinical evaluation of promising hRSV vaccine candidates before proceeding to clinical trials in seronegative infants, hRSV models in non-human primates should be considered for testing the immunopathological safety. We have previously developed a vaccination and challenge model in infant cynomolgus monkeys, in which we reproduced components of the FI-hRSV-mediated immunopathology [128].

BBG2Na is an hRSV subunit vaccine based on a recombinant prokaryote-expressed protein [143]. It consists of the central conserved domain of the hRSV attachment G protein (G2Na, amino acids 130-230) fused to an albumin-binding region (BB) of the streptococcal protein G, and is formulated in alum. BBG2Na was shown to induce protective immune responses against hRSV subgroups A and B in different animal models, without evidence of FI-hRSV-like enhanced immunopathology [143,157-160]. Furthermore, BBG2Na induced protective immune responses in 1-week-old mice, even in the presence of high levels of hRSV-A-specific maternal antibodies [161]. Evaluation of BBG2Na in a phase I/II study in healthy young adults showed that it was safe, well tolerated and highly immunogenic [137].

In the present study, we have evaluated the safety and efficacy of BBG2Na in hRSV seronegative infant macaques, in comparison with animals vaccinated with FI-hRSV also formulated in alum. As non-hRSV vaccinated controls we used animals vaccinated with BB or a wild-type modified vaccinia virus Ankara (MVA-wt), which was included as part of a parallel study on the efficacy and safety of recombinant MVA expressing the hRSV F and G genes. All animals were vaccinated twice, and were challenged four months later intra-tracheally with a macaque-passaged hRSV strain. Emphasis of the study was on the characterization of specific humoral and cellular immune responses after vaccination and challenge and monitoring of previously identified correlates of FI-hRSV-mediated immunopathology.

Materials & Methods

Study design

Ten infant cynomolgus monkeys (*Macaca fascicularis*, age 31 ± 18 weeks) were vaccinated with BBG2Na (n=4), BB (n=2), MVA-wt (n=2) or FI-hRSV (n=2). All animals received two vaccinations at a four-week interval and EDTA blood samples were collected at 0, 2, 4, 6, 8, 12 and 22 weeks after the first vaccination. Heat-inactivated (HI; 30 min, 56°C) plasma samples were stored at -20°C. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and stored at -135°C. Animals 1-4 were vaccinated intra-muscularly (i.m.) with BBG2Na (100µg dose) formulated in Adju-phos (as used by CIPF in clinical trials). Animals 5-6 were vaccinated i.m. with BB (66µg dose) formulated in Alhydrogel (kindly provided by Superfos Biosector, Frederikssund, Denmark). The different adjuvantia were chosen on basis of the differences in isoelectric points of the two proteins, see Dagouassat *et al.* [239]. Animals 7-8 were vaccinated with MVA-wt (provided by B. Moss, NIH, Bethesda, USA). Each animal received a dose of 2×10^8 plaque forming units, which was divided over two anatomical sites: half the dose was administered i.m., the other half intra-nasally (analogous to Stittelaar *et al.* [229]).

Animals 9-10 were vaccinated i.m. with FI-hRSV (10 μg dose) formulated in Adju-phos (Superfos Biosector), prepared as described before [128].

Antibody responses

BBG2Na-, BB-, G2Na-, $G_{172-187}$-, $G_{144-159}$-, $G_{164-176}$- and $G_{190-204}$-specific indirect IgG ELISA's were performed as described previously [137]. hRSV-specific IgG responses were measured in an indirect ELISA (Genzyme Virotech GmbH, Rüsselsheim, Germany), according to the manufacturer's protocol.

Virus neutralizing (VN) antibody responses were measured as previously described [137]. Briefly, serial two-fold dilutions (2^{-3} to 2^{-14}) of HI plasma samples were incubated with approximately 100 $TCID_{50}$ of hRSV (Long strain) for one hr at 37°C, HEp-2 cells were added and cytopathic effect was monitored during the subsequent seven days. 50% VN-titers were calculated from triplicate cultures using the Reed and Muench method [240].

Cellular immune responses

PBMC were thawed, washed and counted, and cultured in RPMI1640 (Biowhittaker, Verviers, Belgium) supplemented with penicillin (100 U/ml; BioWhittaker), streptomycin (100 μg/ml; BioWhittaker), L-glutamin (2mM; BioWhittaker), 2-mercapto-ethanol (10^{-5}M; Merck KGaA, Darmstadt, Germany), 10% HI foetal bovine serum (FBS; Greiner, Frickenhausen, Germany) and 1% HI pooled monkey serum, in 96-well round-bottom plates (Greiner) at a concentration of 1.5 x 10^5 cells per well. Triplicate wells were cultured in the presence of medium alone, BB (10μg/ml), G2Na (5μg/ml) or BBG2Na (10μg/ml). At day 4 supernatants were harvested and cytokine responses (IL-2, IFN-γ, IL-5 and IL-13) were measured using macaque-specific ELISA systems (U-Cytech, Utrecht, The Netherlands) according to the manufacturer's protocol. The detection limits of these assays were 122, 40, 46 and 12 pg/ml, respectively. Subsequently, medium containing ^{3}H-thymidine (0.5μCi per well) was added. Cultures were harvested the next day and cell-associated radioactivity was measured.

hRSV challenge

Eighteen weeks after the second vaccination all animals were challenged intra-tracheally with a macaque-passaged wild-type hRSV A strain (10^6 $TCID_{50}$ [128]). EDTA-blood samples were collected at days –6, 0, 3, 6, 9, 13, 17, 23, 37, 52 and 72 after challenge. Broncho-alveolar lavage (BAL)-samples and throat swabs were collected six days before the challenge, and at days 3, 6, 9, 13 and 72 after challenge. Throat swabs were stored at -70°C. BAL samples were processed for cytospins and RT-PCR. Cytospin slides were later stained (May-Grünwald-Giemsa) and differential cell counts were obtained by light microscopy with 1,000 cells per slide counted.

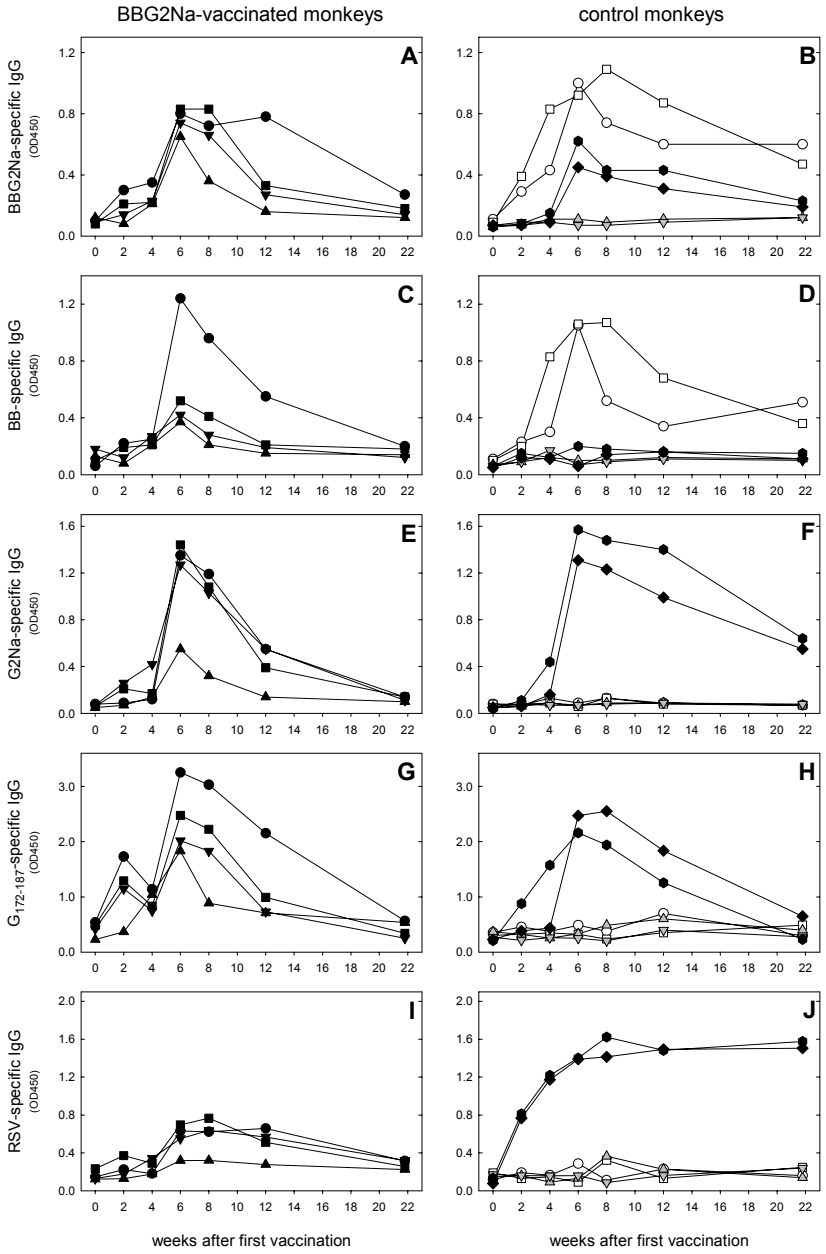

Figure 1: BBG2Na- (A and B), BB- (C and D), G2Na- (E and F), $G_{172-187}$- (G and H) and hRSV-specific (I and J) IgG responses in BBG2Na-vaccinated (A, C, E, G and I) or control (B, D, F, H and J) animals. Symbols representing the BBG2Na-vaccinated animals are circle, square, triangle up and triangle down for animals #1, #2, #3 and #4, respectively. For the control animals, white symbols indicate BB-vaccinated animals, grey symbols MVA-wt-vaccinated animals and black symbols FI-RSV-vaccinated animals. Symbols representing the control animals are circle, square, triangle up, triangle down, diamond and hexagon for animals #5, #6, #7, #8, #9 and #10, respectively.

Real-time hRSV-specific RT-PCR

For RT-PCR, 5 x 10^5 BAL cells were frozen as a dry pellet at -70°C. RNA was isolated using the High Pure viral RNA kit (Roche Diagnostics, Almere, The Netherlands). hRSV A was specifically detected and semi-quantified using a real-time detection assay. RNA was amplified using an EZ RT-PCR amplification kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) on an ABI7000 sequence detection system. Briefly, 10µl of RNA was amplified in a total volume of 50µl using 45 pmol of each of the primers hRSV-A forward 5'-AGATCAACTTCTGTCATCCAGCAA-3' and hRSV-A reverse 5'-TTCTGCACATCATAATTAGGAGTATCAAT-3', and 5 pmol of a FAM labelled probe 5'-CACCATCCAACGGAGCACAGGAGAT-3'. After initial reverse transcription (2 min at 50°C, 30 min at 60°C) followed by an UNG inactivation step of 5 min at 95°C, the cDNA was amplified for 40 cycles of 20 s at 94°C and 1 min at 62°C. The cycle threshold (Ct) value was calculated automatically when the FAM-specific hRSV-A signal was above the background and used to get a semi-quantitative impression of the hRSV viral load. All reactions contained both negative as well as a low positive controls.

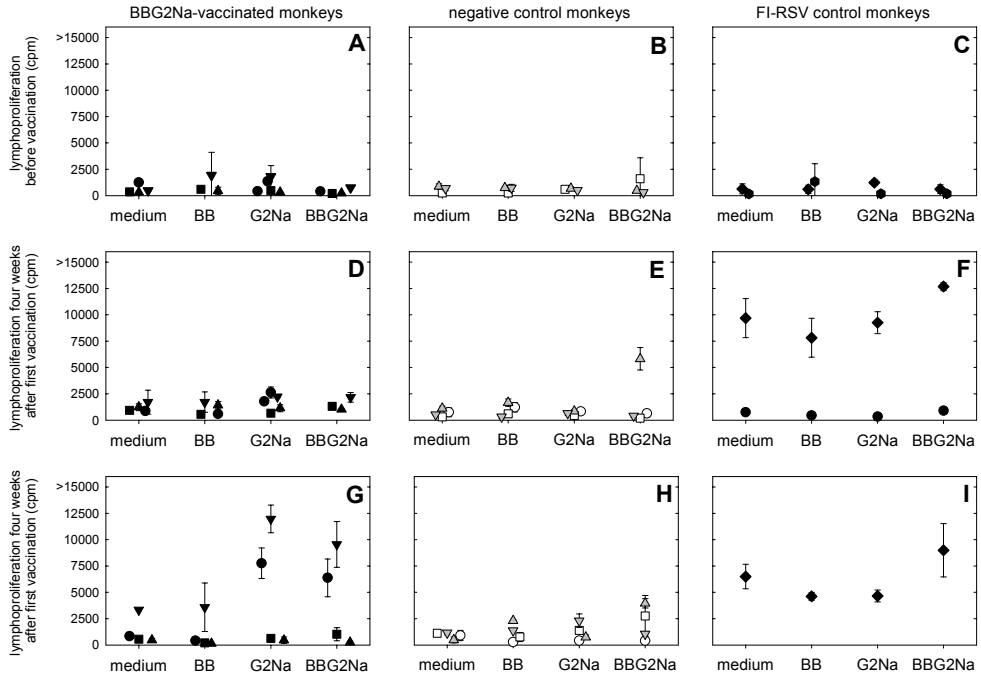

Figure 2: Proliferative responses of PBMC after stimulation with medium, BB, G2Na or BBG2Na before vaccination (A-C), four weeks after the first vaccination (D-F) or four weeks after the second vaccination (G-I). Symbols and symbol fills are identical to figure 1.

Results

Humoral immune responses after vaccination

Induction of BBG2Na-specific IgG responses was detected in all animals vaccinated with BBG2Na, BB or FI-hRSV, but not in animals vaccinated with MVA-wt (figure 1A-B). Induction of BB-specific IgG responses was detected in all animals vaccinated with BBG2Na or BB, but not in animals vaccinated with MVA-wt or FI-hRSV (figure 1C-D). Induction of IgG responses to the hRSV-specific component of the BBG2Na vaccine, G2Na, was detected in all animals vaccinated with BBG2Na or FI-hRSV, but not in animals vaccinated with BB or MVA-wt (figure 1E-F). Of the four G2Na-peptides tested, induction of peptide $G_{172\text{-}187}$-specific IgG responses was detected in all animals vaccinated with BBG2Na or FI-hRSV, but not in animals vaccinated with BB or MVA-wt (figure 1G-H). In one of the animals (animal #2) vaccinated with BBG2Na, and in both FI-hRSV-vaccinated animals, IgG responses to peptide $G_{144\text{-}159}$ were also detected (results not shown). Peptide $G_{164\text{-}176}$- or $G_{190\text{-}204}$-specific IgG responses could not be

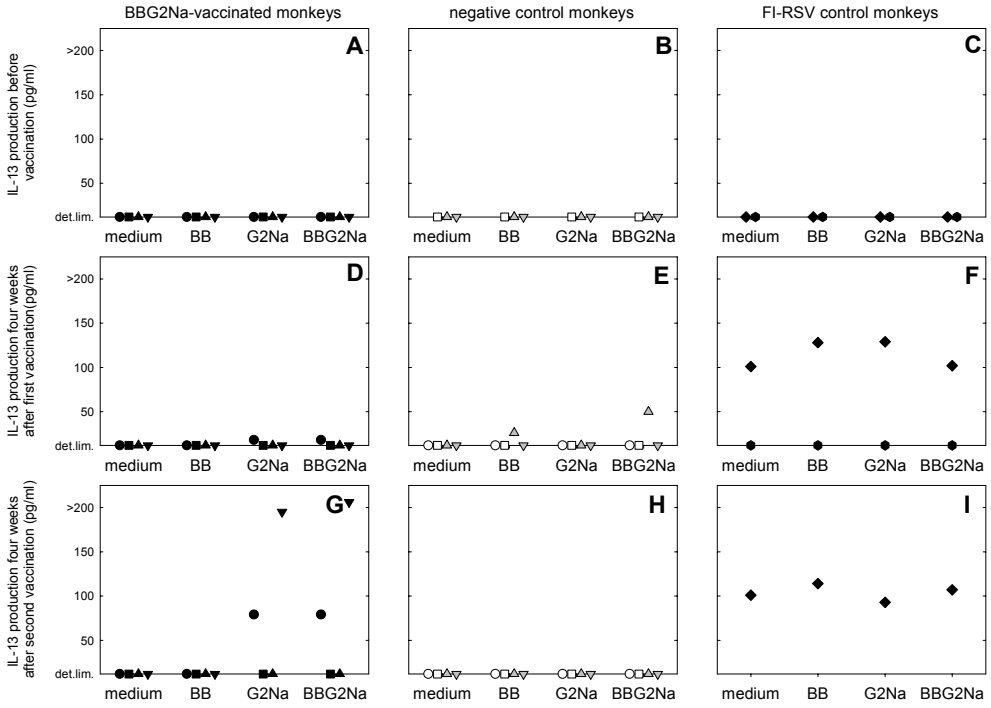

Figure 3: IL-13 levels in supernatants of PBMC cultures shown in figure 2. PBMC collected before vaccination (A-C), four weeks after the first vaccination (D-F) or four weeks after the second vaccination (G-I) were stimulated with medium, BB, G2Na or BBG2Na. Symbols and symbol fills are identical to the previous figures.

detected in any of the animals (results not shown). Induction of hRSV-specific IgG responses was detected in three out of four animals vaccinated with BBG2Na (animals #1, #2 and #4), in both animals vaccinated with FI-hRSV, but not in animals vaccinated with BB or MVA-wt (figure 1I-J). Induction of hRSV-specific virus neutralizing (VN) antibodies could only be shown in animals vaccinated with FI-hRSV (results not shown).

Cellular immune responses after vaccination

Four weeks after the second vaccination G2Na- and BBG2Na-specific proliferative PBMC responses were detected in two (animals #1 and #4) of four animals vaccinated with BBG2Na (figure 2G). In addition, specific proliferative responses were detected in one of the two BB-vaccinated animals (animal #6, see figure 2E), while no specific responses were detected in the other animals tested either before or after vaccination (figure 2). G2Na- and BBG2Na-specific IL-13 was detected in culture supernatants of the same two responding animals four weeks after the second vaccination but not before or four weeks after the first vaccination (figure 3G, A and D,

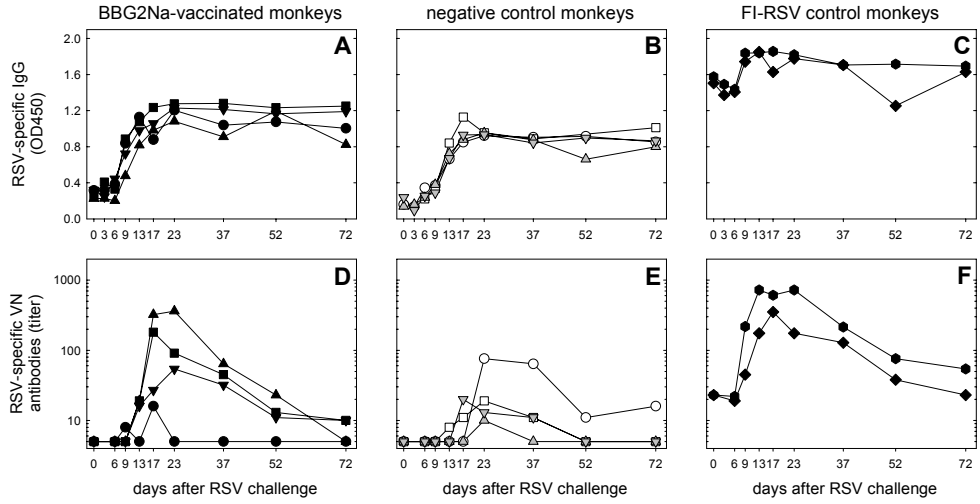

Figure 4: hRSV-specific IgG (A, B and C) and VN antibody (D, E and F) responses after hRSV challenge in BBG2Na-vaccinated (A and D) or control (B, C, E, F) animals. Symbols and symbol fills are identical to the previous figures. The hRSV-specific IgG responses of the BBG2Na-vaccinated (panel A) and the control (panel B) animals were significantly different ($p<0.01$, mixed model ANOVA).

respectively). In these supernatants IL-5 (G2Na: 96 and 200 pg/ml; BBG2Na: 138 and 163 pg/ml for animals #1 and #4, respectively) and IL-2 (G2Na: 147 and 180 pg/ml; BBG2Na: undetectable and 189 pg/ml for animals #1 and #4, respectively), but not IFN-γ, were also detected, which were not present in the supernatants of medium controls or BB-stimulated PBMC. IL-13 was also detected in the supernatant of BB and BBG2Na-stimulated PBMC of BB-vaccinated animal #6 (figure 3E), but not in supernatants of PBMC

collected from the other animals vaccinated with BB or MVA-wt (figure 3 and results not shown).

In culture supernatants of the FI-hRSV-vaccinated animals collected four weeks after the first vaccination IL-13 was detected in supernatant of PBMC collected from animal #9, but not animal #10. Four weeks after the second vaccination we were only able to test supernatant from animal #9, which resulted in a similar picture as on the previous sampling point. In all cultures from FI-hRSV-vaccinated animals production of IL-13 was largely irrespective of the *in vitro* stimulation (figure 3F and I and results not shown). However, in both FI-hRSV-vaccinated animals higher IL-13 levels could be detected in the supernatants of PBMC stimulated *in vitro* with beta propiolactone (BPL)-inactivated hRSV than in those stimulated with a BPL-inactivated Vero cell control antigen (486 pg/ml and 111 pg/ml versus 104 pg/ml and less than 12 pg/ml for animals #9 and #10, respectively). Upon stimulation of PBMC obtained from BBG2Na-vaccinated animals with the same antigens, IL-13 was again only detectable in the supernatants of animals #1 (30 pg/ml) and #4 (59 pg/ml). In the FI-hRSV-vaccinated animals hRSV-specific IL-5 (n=2) and/or IL-2 (n=1: animal #9), but not IFN-γ, were detected. These cytokines were undetectable in all BBG2Na-, BB- or MVA-wt-vaccinated animals.

hRSV challenge

Eighteen weeks after the second vaccination all animals were challenged intra-tracheally with hRSV. At this moment only the FI-hRSV-vaccinated animals still had detectable hRSV-specific serum antibodies (figure 4A-C). In the other animals hRSV-specific IgG responses were rapidly induced, with the BBG2Na-vaccinated animals responding earlier and reaching slightly higher values than the BB- or MVA-wt-vaccinated control animals ($p<0.01$, mixed model ANOVA, figure 4A-B). hRSV-specific IgG responses were also boosted in the FI-hRSV-vaccinated animals, although this effect could not optimally be shown at the 1:100 plasma dilution used according to the ELISA manufacturer's instructions (figure 4C). The ELISA was later repeated using a 1:1000 plasma dilution, resulting in a curve similar to the virus neutralization response shown in figure 4F (not shown).

All animals also showed a transient VN antibody response upon challenge, again with a tendency of slightly earlier and higher responses in the BBG2Na-vaccinated animals (no significant difference, mixed model ANOVA, figure 4D-E). Interestingly, the animals with detectable vaccine-induced cellular immune responses (#1 and #4) had lower VN antibody responses than the other two BBG2Na-vaccinated animals. The two FI-hRSV-vaccinated animals also showed a rapid but transient VN antibody booster response after challenge (figure 4F).

The experimental design was not optimized to enable hRSV re-isolation from BAL cells. As an alternative approach to monitor pulmonary virus loads, an hRSV-specific real-time RT-PCR was performed on BAL cells collected before and after challenge. As shown in figure 5A-C, no significant

differences in pulmonary virus loads could be demonstrated. These results were confirmed in another semi-quantitative RT-PCR performed as described previously ([128], results not shown). hRSV isolations in HEp-2 cells and hRSV-specific RT-PCR on frozen throat swab samples were all negative (results not shown). Virus isolations were not performed on BAL cells due to limitations in sample quantity.

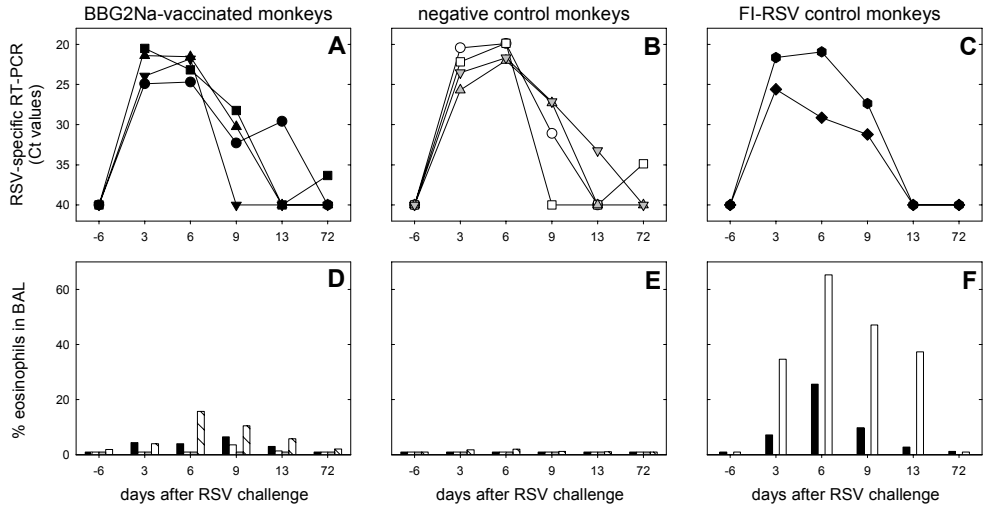

Figure 5: hRSV-specific real-time RT-PCR signals (A-C) and percentages of eosinophils (D-F) in BAL samples collected at different time points after hRSV challenge. Symbols and symbol fills are identical to the previous figures. In panels D and E animals #1 to #4 and #5 to #8 are represented by bars with black, white, grey or hatched fills, respectively. In panel F animals #9 and #10 are represented by bars with black and white fills, respectively.

Cytospins were prepared of BAL cells collected before and after challenge, and eosinophil percentages were determined by differential counting. In accordance with our previous results, high eosinophil percentages were detected in both FI-hRSV vaccinated animals (figure 5F), with a peak at 6 days after challenge. In contrast, eosinophils could hardly be detected in the BB- or MVA-wt-vaccinated control animals (figure 5E). In two of four BBG2Na-vaccinated animals (#1 and #4), but not in the other two animals of this group, low percentages of eosinophils were detected after hRSV challenge (figure 5D). Interestingly, these were the same two animals that also showed *in vitro* BBG2Na- and G2Na-induced IL-13, IL-5 and IL-2 responses after vaccination.

Discussion

We have evaluated the immunopathological safety of the hRSV subunit vaccine BBG2Na formulated in alum in seronegative infant macaques, in comparison with FI-hRSV. BBG2Na did not predispose monkeys for

immunopathology similar to FI-hRSV. However, in two of four BBG2Na-vaccinated animals IL-13 producing specific T cells were detected upon vaccination, and in the same two animals a low-level eosinophilia was detected in BAL cells upon hRSV challenge.

Vaccination of infant macaques with BBG2Na induced good G2Na-specific IgG antibody responses in three out of four animals. However, in a commercial hRSV ELISA specific IgG responses were considered in the gray zone according to the kit's cut-off serum, and no hRSV-specific VN antibodies could be detected. This contrasts with data in mice and cotton rats, in which strong hRSV-specific IgG and low to moderate VN antibody responses were observed [143]. Furthermore, the data also differ from the strong although non-neutralizing hRSV-specific IgG responses detected in BBG2Na-vaccinated African green monkeys [241](U.F. Power, personal observations). In a clinical trial in healthy adults we had previously detected VN antibody responses after vaccination with BBG2Na [137], but these boosters of pre-existing immunity are difficult to compare with the primary immune responses described in the present study.

In two of four animals, vaccination with BBG2Na induced vaccine-specific proliferative T cell responses, which proved to be associated with the production of IL-13. This cytokine was previously identified as a correlate of FI-hRSV-mediated immunopathology in this model [128]. This is in accordance with results in BALB/c mice, where vaccination with BBG2Na formulated in alum was found to induce type 2 immune responses [157]. However, in BALB/c mice it was found that these primed responses were not recalled upon hRSV challenge [157,158]. In the present study, we detected eosinophils in the BAL cells of the two BBG2Na-vaccinated macaques that had developed IL-13 producing T cells, although the levels were substantially lower than those found in the two FI-hRSV controls. However, these results could indicate that in these two animals the primed type 2 responses were recalled by the challenge, although it remains questionable if this would have clinical relevance in humans.

Using semi-quantitative RT-PCR analysis, we could not detect significant differences in pulmonary viral loads between BBG2Na-vaccinated and control animals. However, it is debatable if this allows conclusions about the protective capacity of BBG2Na. Macaques are less susceptible to hRSV than humans, which forced us to choose a relatively high challenge dose [128]. In addition, the challenge was given intra-tracheally to ensure delivery of the virus into the lungs, which makes the model artificial when compared to the natural route of hRSV infection. In our previous study, we could detect a partial protection mediated by FI-hRSV immunization by using hRSV-specific semi-quantitative RT-PCR on BAL-cells, but these animals had also developed VN antibody titers upon vaccination [128]. In the present study a similar partial protection was detected in one of two FI-hRSV vaccinated animals, but in none of the BBG2Na-vaccinated animals. To optimize the model for detection of protection, larger numbers of BBG2Na-vaccinated and control animals should be challenged with different doses of hRSV given by

different routes of infection. In addition, where we have largely focused on characterization of specific immune responses, higher priority should be given to re-isolation of the virus from different organs. Studies in non-human primates are usually limited in numbers of animals, due to ethical, practical and financial constraints. Large-scale studies would be justified, however, with a vaccine formulation that is being considered for administration to seronegative infants, and should be combined with monitoring of immunopathology.

In conclusion, BBG2Na formulated in alum induced specific IL-13 responses following vaccination and predisposed for a low level eosinophilia upon hRSV challenge in two out of four animals. Although these data were obtained in a relatively limited number of animals and are in contrast with previous observations in rodents and African green monkeys [143,157-160,241], they indicate that more extensive studies in non-human primates focusing on immunopathological safety would be required before proceeding to clinical trials with BBG2Na in seronegative infants.

Acknowledgements

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Chapter 4.2

Vaccination of infant macaques with a recombinant modified vaccinia virus Ankara expressing the respiratory syncytial virus F and G genes does not predispose for immunopathology.

L. de Waal, L.S. Wyatt, S. Yüksel, G. van Amerongen, B. Moss, H.G.M. Niesters, A.D.M.E. Osterhaus, R.L. de Swart

Vaccine (*in press*)

Abstract

We have evaluated the safety and immunogenicity of a recombinant modified vaccinia virus Ankara (MVA) vector expressing the human respiratory syncytial virus (hRSV) fusion (F) and attachment (G) proteins in infant macaques. Animals were vaccinated twice and four months later challenged with hRSV. Although vaccination did not predispose for immunopathology upon challenge, we were also unable to demonstrate protection. Since vaccination had resulted in priming for secondary immune responses upon challenge, we suggest that vaccination efficacy will have to be improved by using MVA in a prime-boost strategy.

Introduction

We have evaluated the safety and efficacy of two candidate human respiratory syncytial virus (hRSV) vaccines in infant macaques, using a common group of control animals. While the results obtained with BBG2Na were described before [242], we report here the data obtained with a recombinant modified vaccinia virus Ankara vector (rMVA) expressing the hRSV fusion (F) and attachment (G) proteins [156].

Since inactivated hRSV vaccines have been associated with predisposition for enhanced disease in hRSV seronegative individuals, live attenuated vaccines or recombinant viral vectors may represent a safer approach when looking for new candidate hRSV vaccines in infants. Several studies have focused on the development of a live attenuated hRSV vaccine, although it has been difficult to find a proper balance between attenuation and immunogenicity [130]. As an alternative, live vectors mediating the expression of hRSV genes have been considered [131-134,136]. MVA is a replication-deficient poxvirus, which was used in the late stage of the smallpox eradication campaign. When used as a vector, it induced similar expression levels of the recombinant genes as compared to the fully competent strains of vaccinia virus (VV) [162,163], and induced equal or better humoral and cellular immune responses in animals [164-166]. The fact that MVA is replication-deficient in mammalian cells ensures safe usage of this vector, as has been described in a safety study in immunocompromised macaques [167]. In addition, the vaccination dose can be relatively high, which would enable breaking the maternal antibody barrier in young infants.

Vaccination of mice with recombinant MVA expressing the hRSV transmembrane glycoproteins F, G or both F and G resulted in strong specific antibody responses and protection from challenge [156]. In the present study, we have vaccinated infant macaques twice with a combination of the two single recombinants (rMVA-F and rMVA-G), using animals vaccinated with wild-type MVA (MVA-wt), BB (the albumin-binding domain of streptococcal protein G) or FI-hRSV as controls as described earlier [242]. All animals were challenged intra-tracheally with a macaque-passaged hRSV

strain four months after the second vaccination. The induction of humoral and cellular immune responses by vaccination and challenge was monitored, with special emphasis on the previously described *ex vivo* and *in vitro* correlates of FI-hRSV-mediated immunopathology.

Materials & Methods

The study design of this experiment has been described previously [242]. In addition to the ten animals described, four infant cynomolgus monkeys (*Macaca fascicularis*) were vaccinated with a combination of rMVA-F and rMVA-G (rMVA-F/G). These animals, which are referred to as animals M1 to M4, were vaccinated with a dose of 2 x 10^8 plaque forming units, of which half was administered intra-muscularly and half intra-nasally. The vaccinations were given twice with an interval of four weeks.

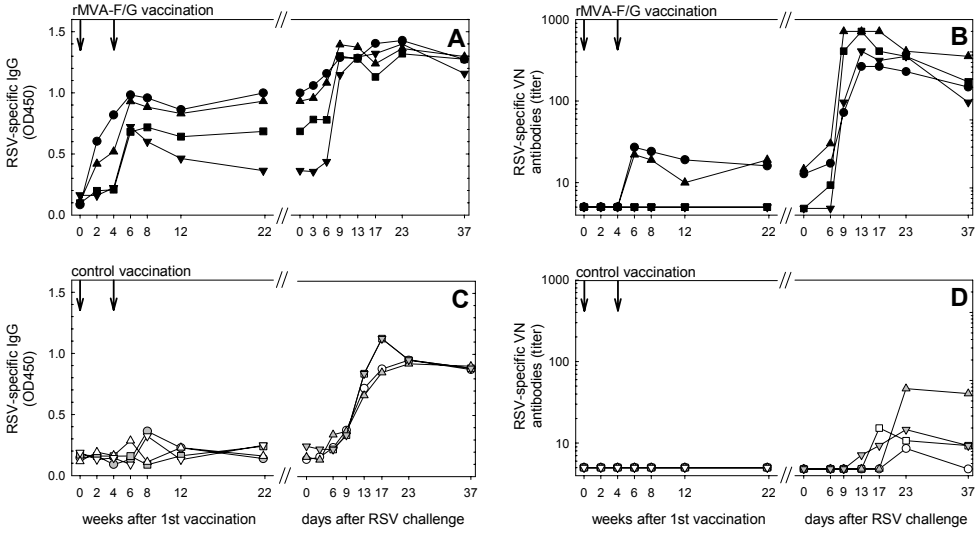

Figure 1: hRSV-specific IgG (A and B) and VN antibody (C and D) responses in macaques after vaccination with rMVA-F/G (A and C) or control preparations (B and D). In each panel responses after vaccination and challenge are shown on the left and right, respectively. Arrows indicate times of vaccination. Symbols representing the rMVA-F/G animals are circle, square, triangle up and triangle down for animals M1, M2, M3 and M4, respectively. Symbols representing the MVA-wt-vaccinated animals are white circle and square, BB-vaccinated animals gray triangle up and triangle down.

hRSV-specific IgG responses were measured in an indirect ELISA (Genzyme Virotech GmbH, Rüsselsheim, Germany), according to the manufacturer's protocol. Virus neutralizing (VN) antibody responses were measured as previously described [137]. Briefly, serial two-fold dilutions (2^{-3} to 2^{-14}) of HI plasma samples were incubated with approximately 100 $TCID_{50}$ of hRSV (Long Strain) for one hour at 37°C, HEp-2 cells were added and cytopathic effect was monitored during the subsequent seven days. 50% VN-

titers were calculated from triplicate cultures using the Reed and Muench method [240].

Eighteen weeks after the second vaccination all animals were challenged intra-tracheally with hRSV [242]. Pulmonary virus loads were monitored using hRSV-specific real-time RT-PCR using 5 x 10^5 broncho-alveolar lavage (BAL) cells [242]. In addition, cytospin slides were prepared from BAL cells, stained (May-Grünwald-Giemsa) and differential cell counts were obtained by light microscopy with 1,000 cells per slide counted.

Results

Vaccination

Using an immunofluorescence method as described previously [229], induction of vaccinia virus-specific IgG responses was detected in all animals vaccinated with rMVA-F/G or MVA-wt, but not in the controls (results not shown). Induction of hRSV-specific IgG antibodies was detected in all animals vaccinated with MVA-F/G, although responses in two of the four animals (M2 and M4) were considered gray zone according to the kit's cut-off serum, while the other two animals (M1 and M3) were considered positive (figure 1A). hRSV-specific VN antibodies were only detected in two of these four animals (M1 and M3, figure 1B) and in both animals vaccinated with FI-hRSV (not shown). Low hRSV-induced proliferative responses were detected in animals M1 and M4, but none of these responses were accompanied by detectable levels of interleukin (IL)-13, IL-5, IL-2 or interferon-γ in their supernatant (not shown).

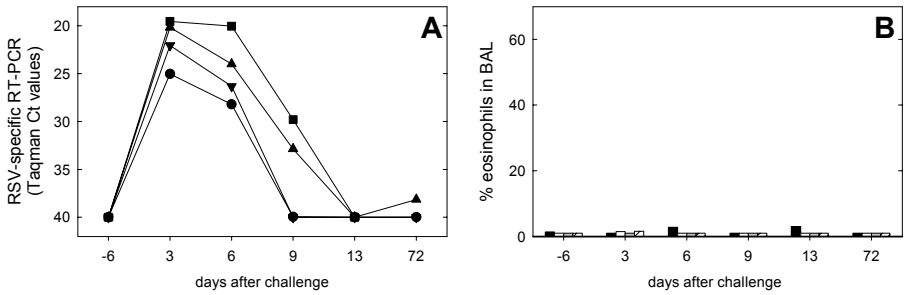

Figure 2: hRSV-specific real-time RT-PCR signals (A) and percentages of eosinophils (B) in BAL samples collected at different time points after hRSV challenge. Symbols representing the rMVA-F/G animals are circle, square, triangle up and triangle down for animals M1, M2, M3 and M4, respectively.

hRSV challenge

At the moment of challenge, all rMVA-F/G-vaccinated animals still had detectable hRSV-specific serum antibodies (figure 1A). After challenge, hRSV-specific IgG and VN antibody responses were rapidly boosted, with the rMVA-F/G-vaccinated animals responding earlier and reaching higher levels than the control animals (figure 1A and B).

As shown in figure 2A, pulmonary virus loads were not lower than those measured in the control or BBG2Na-vaccinated animals [242]. Similar to the control animals, low numbers of eosinophils were detected in BAL samples (figure 2B), while high numbers had been detected in both FI-hRSV-vaccinated animals [242].

Discussion

In the present study we have addressed the immunological safety and efficacy of recombinant MVA encoding the F- and G-genes of hRSV in seronegative infant macaques, in comparison with FI-hRSV. Vaccination with rMVA-F/G did not induce the previously described correlates of FI-hRSV-mediated immunopathology, but we were also unable to demonstrate vaccination-mediated protection.

Vaccination of macaques with rMVA resulted in comparable levels of vaccinia virus-specific antibodies as observed in a previous study in our lab, in which juvenile macaques were vaccinated with rMVA expressing the measles virus F and H genes [229]. However, whereas in the measles study high measles virus-specific VN antibody titers were induced, vaccination with rMVA-F/G induced low or undetectable hRSV-specific VN antibody levels. In contrast, Wyatt *et al.* [156] had shown that vaccination of mice with this construct resulted in high levels of hRSV-specific antibodies.

In addition to the absence of detectable proliferative responses or type 2 cytokine production by PBMC, priming with rMVA-F/G also did not result in pulmonary eosinophilia or neutrophilia (not shown) upon hRSV challenge. This suggests that the hRSV-specific immune responses induced by vaccination did not predispose the animals for immunopathology similar to that induced by FI-hRSV.

Despite two consecutive vaccinations with rMVA-F/G we were unable to demonstrate protection from challenge with a macaque-passaged hRSV strain. This might be related to the use of a relatively high challenge dose (10^6 $TCID_{50}$). The absence of rMVA-F/G-mediated protection could also be related to the young age of the animals used. Whereas Stittelaar *et al.* [229] were able to show protection from measles challenge after vaccination with MVA-FH in juvenile monkeys (3-4 years old), Zhu *et al.* [243] were unable to show protection after vaccination with the same construct in infant monkeys (1-2 weeks old). All rMVA-F/G-vaccinated animals showed strong secondary antibody responses when compared to the controls, demonstrating that vaccination had successfully primed the immune system. However, the hRSV-specific VN antibody response appeared between days 6 and 9 after challenge, which is just after the peak of the hRSV replication. Therefore, although the vaccine was unable to prevent hRSV infection, it would probably prevent or limit the extent of severe hRSV bronchiolitis when applied in infants.

In future experiments, the possibility to improve the efficacy of rMVA-F/G vaccination could be evaluated by the application of different

vaccination schemes, or combination with other vaccine candidates in prime-boost regimens [244,245].

Acknowledgements

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Chapter 5

Summarizing Discussion

To date, only few studies have been performed to characterize the human respiratory syncytial virus (hRSV)-specific cellular immune response in humans. The experiments described in the present thesis were carried out to evaluate the role of specific T cells in protection from and immunopathogenesis of hRSV infection. In chapter 2, the hRSV-specific T cell response was studied in infants with or without laboratory-confirmed hRSV infections of different clinical severity. In chapter 3, the hRSV-specific T cell response was investigated in infants and healthy young adults. HLA class I- and class II-restricted T cells were studied at a clonal level and in bulk cultures. In chapter 4, the safety and efficacy of two candidate hRSV vaccines were evaluated in a vaccination/challenge model in macaques.

An immunological link between hRSV infection and atopic disease?

Several studies have suggested a relationship between hRSV infections in early life and the development of atopic disease such as asthma and allergy [98,99]. However, whether this relationship is causal, or results from a common physiological predisposition of certain infants continues to be subject of debate [194,195]. On the one hand, it has been hypothesized that a skewed virus-specific T cell response associated with the production of Th2-like cytokines such as IL-4, IL-5 and IL-13 could influence maturation of the immune system [246]. On the other hand, infants with a family history of atopy (FHA) have been shown to have an increased risk to develop atopic sensitization [98], and these infants may also be more prone to severe hRSV infections.

A prospective cohort study entitled "Viral infections in the immunopathogenesis of allergy in early childhood" (in Dutch abbreviated as Vigall) was performed by the Erasmus MC departments of Pediatrics, Otorhinolaryngology and Virology. The aim of Vigall was to evaluate the causal relationship between viral respiratory infections and the development of the immune system and the clinical expression of allergic disease in infants. To this end, clinical samples were collected from infants with or without FHA at the age of 6, 12, 18 and 24 months, and during the acute and convalescent phase of each upper respiratory tract infections (URTI) during their first two years of life.

Different approaches were used to assess epidemiological, virological and immunological characteristics of the infants in the Vigall cohort. Results of these studies were also described in the following PhD theses [247,248].

In the present thesis, the clinical samples obtained in the Vigall project were used to characterize the RSV-specific cellular immune response, both local and systemic, upon laboratory-confirmed hRSV infection. Unfortunately, in 129 infants only twenty hRSV-induced URTI and two lower respiratory tract infections (LRTI) were identified, almost exclusively in infants with FHA. As a result, it became virtually impossible to study the hRSV-specific immune response in relation to genetic predisposition. Therefore, we decided to investigate, with the available samples, the hRSV-

specific T cell response in infants with hRSV- or non-hRSV-induced URTI, in comparison with infants hospitalized with hRSV-induced LRTI.

hRSV-specific T cells: a role in severity of hRSV infection?

A number of studies have evaluated why the majority of infections cause limited or no clinical signs, while a small subset of the infections is associated with severe disease. A first possible explanation is that some individuals could have a physiological predisposition, e.g. in the form of a low maternally-derived VN antibody titer or a pre-existent lung function abnormality [81]. Secondly, the infectious dose may be important [82], and also concurrent infection with other agents could result in enhancement of disease [83]. Finally, it has been hypothesized that the hRSV-specific immune response itself may contribute to the severity of the disease. In other words, certain manifestations of hRSV-mediated disease are of immunopathological nature. The latter hypothesis is often associated with the hypothesis that hRSV-mediated LRTI during early life could induce, or be a marker for, an atopic asthmatic phenotype [84]. However, results from the collective studies addressing the phenotype of T cells during hRSV-mediated LRTI are conflicting [87-97].

In the study described in **chapter 2**, significantly higher frequencies of specific T cells were detected in the infants hospitalized with hRSV bronchiolitis than in infants with mild hRSV-mediated URTI or non-hRSV-mediated URTI. However, the infants in the bronchiolitis group were significantly younger than those in the two URTI groups, prohibiting a direct comparison of these responses. Detection of Th1 and Th2 cytokine producing cells in PBMC and nasal T cells showed that there were few qualitative differences between these three groups: all infants showed a mixed Th1/Th2 response upon infection, without significant differences between the groups.

However, IL-13 producing hRSV-specific T cells were mainly detected in samples collected from infants with severe hRSV LRTI, during the convalescent phase of infection. This cytokine could be the link between more severe hRSV infections at young age and the subsequent exacerbation of allergic asthma at later age, since IL-13 appeared to play a central role in the pathogenesis of asthma and atopic disease [110-113,197]. Recurrent hRSV infections in infants producing IL-13 upon infection could lead to boosting the IL-13 response after each infection, eventually leading to an increase of IL-13 producing T cells and the development of asthma. However, large-scale prospective studies will be required to test this hypothesis.

hRSV-specific T cells: analysis of clones and bulk cultures

In the studies described in **chapter 3**, two approaches were used to study the hRSV-specific T cell response. The first approach was to identify hRSV-specific HLA class I- and class II-restricted T cell epitopes by limiting

dilution analyses. To this end, T cell lines were enriched for specific T cells by stimulation with autologous hRSV-infected B-lymphoblastic cell lines (BLCL-hRSV). IFN-γ ELISPOT assays were used as a read-out for specific responses.

In **chapter 3.1**, $CD8^+$ HLA class I-restricted T cell clones (TCC) from two infants were generated four weeks after a severe laboratory-confirmed hRSV infection. Two different hRSV-specific CD8-TCC were generated from each patient, with specificities for the hRSV F and 1B or the F and 1C protein, respectively. The F-specific CD8-TCC were selected for further characterization using a set of overlapping peptides. Both CD8-TCC were found to recognize minimal epitopes of nine amino acids: CD8-TCC-1 recognized $F_{118\text{-}126}$ (RARRELPRF) and was restricted over HLA-B*57, while CD8-TCC-2 recognized $F_{551\text{-}559}$ (IAVGLLLYC) and was restricted over HLA-C*12.

In **chapter 3.2**, two $CD4^+$ HLA class II-restricted TCC were generated from nasal brush cells or PBMC collected from two infants during the acute or convalescent phase of infection, respectively. CD4-TCC-1 was specific for the matrix (M) protein, recognized a minimal epitope of nine amino acids and was restricted over HLA-DPB1*1601. The minimal epitope of this clone was either $M_{247\text{-}255}$ (TRFAIKPME) or $M_{248\text{-}256}$ (RFAIKPMED). CD4-TCC-2 was specific for the G protein, recognized a minimal epitope of nine amino acids ($G_{163\text{-}171}$, FHFEVFNFV), and was restricted over HLA-DPB1*0401 and 0402.

Interestingly, the G-specific CD4-TCC recognized an epitope located in the central conserved domain of the hRSV G protein (conserved in both hRSV group A and B viruses), and its restriction element HLA-DP4 has a high global allelic frequency [236]. Since in mouse models of hRSV disease it has been suggested that G-specific MHC class II-restricted T cells may play a role in disease enhancement [64,191], we set out to test the hypothesis that the ability to present this epitope could be a factor in determining whether some infants develop more severe hRSV disease than others. However, functional studies using BLCL from infants with mild or severe hRSV-induced LRTI as APC to the G-specific CD4-TCC suggested that severity of disease is not linked to the allelic frequency of HLA-DP4.

Whether the frequency of $G_{163\text{-}171}$-specific T cells influences the outcome or hRSV disease remains to be determined. Studies addressing this assumption should be performed *ex vivo* to exclude phenotype or specificity changes due to culturing or stimulating hRSV-specific T cells. Since it is difficult to perform large scale *ex vivo* T cell studies on patient materials from infants with mild or severe hRSV disease, due to sample size, a way out for this problem would be the use of tetramers in combination with the G-specific HLA-DP4-restricted epitope. However, while the use of tetramer-peptide complexes for HLA class I-restricted T cells is widely accepted and used [249,250], the use of HLA class II tetramer-peptide complexes is still limited and mainly based on the HLA-DRB1* and -DQB1* alleles [251-254]. It would be of interest to use tetramers based on the HLA-DPB1*0401

or 0402 alleles to study the frequency of peptide-specific T cells in infants with either mild or severe LRTI.

From an evolutionary point of view, the presence of a conserved T cell epitope restricted by the highly frequent HLA-DP4 is difficult to understand. For influenza viruses and other viruses, it has been described that point mutations in an epitope can result in escape from HLA-binding or T cell recognition, resulting in a selective growth advantage for the virus [255]. The fact that the HLA-DP4-restricted epitope is located in the central conserved region of the G protein could mean that mutations in this epitope are restricted by conformational constraints and would alter the function of the G protein or even lead to loss of function. Even so, the resulting immunological pressure from the response to this epitope in the majority of the human population has been insufficient to reduce or prevent circulation of hRSV. In contrast, if the $G_{163\text{-}171}$-specific T cell response would play a role in the pathogenesis of hRSV and would be related to the induction of severe disease, it could be hypothesized that the circulation of hRSV in the human population would have caused a negative selection pressure on the allelic frequency of HLA-DP4. The result of this putative selection pressure is not reflected by the high allelic frequency of HLA-DP4.

In **chapter 3.3** T cell responses directed to the hRSV transmembrane glycoproteins F and G were studied in PBMC bulk cultures, after enrichment by stimulation with autologous cells infected with recombinant modified vaccinia viruses (rMVA) mediating the expression of F (rMVA-F) or G (rMVA-G). Using this approach two different questions could be posed as addressed below: how diverse is the epitope usage of the hRSV F-specific T cell response, and does the hRSV G protein contain HLA class I-restricted CTL epitopes?

HLA class II-restricted F-specific T cell responses are highly diverse

Previous studies [67] and those described in **chapter 3.1** of this thesis identified three HLA class I-restricted CTL epitopes in the hRSV F protein. F-specific HLA class II-restricted memory T cell responses have been studied in bulk cultures using overlapping peptides spanning the F protein [70,71]. These studies identified a number of antigenic regions containing T cell epitopes. The approach used in **chapter 3.3** of the present thesis using enrichment of specific T cells by stimulation with autologous rMVA-F-infected cells resulted in identification of both $CD4^+$ and $CD8^+$ T cell responses, although the majority of specific T cell lines contained F-specific $CD4^+$ T cells. Many of the antigenic regions identified overlapped with those described previously, although a number were newly identified as shown in figure 1. This figure also clearly illustrates that especially the Th epitope-containing regions cover almost the complete F sequence.

Although precursor frequencies were not determined, in donor 3 eleven F-specific T cell lines were obtained from eleven original rMVA-F-stimulated wells containing 50,000 PBMC each. These T cell lines displayed

responses to ten different antigenic regions, suggesting that the frequency of these precursor cells in PBMC may be in the order of magnitude of $1:10^4$ - $1:10^5$. If the precursor frequencies would be substantially higher, it could be expected that T cells of one specificity (or at least a restricted number of specificities) would have been found in each culture. This assumption is supported by the fact that stimulations with the positive peptides in PBMC immediately followed by IFN-γ ELISPOT did not result in detectable specific responses using 150.000 cells per well (results not shown).

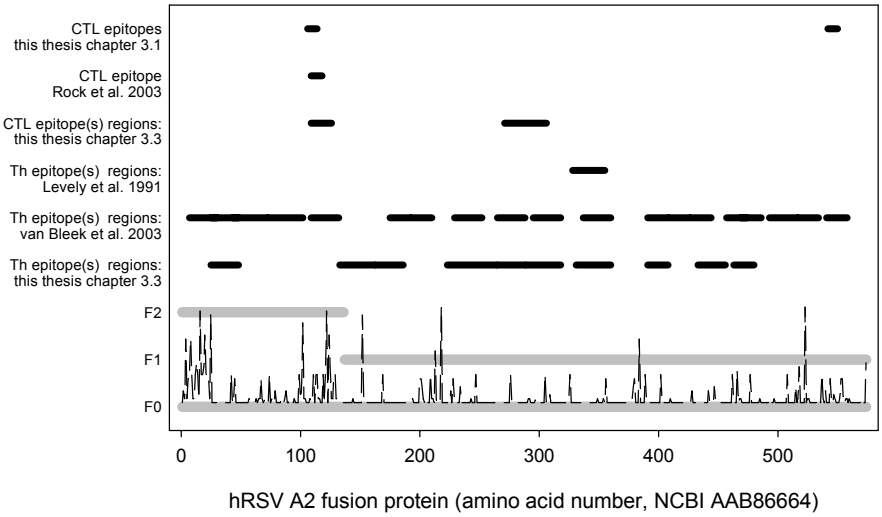

Figure 1: graphical representation of T cell epitopes or epitope(s) containing regions in the hRSV F protein. Gray lines represent the hRSV A2 F0 precursor-protein and its subunits F1 and F2. The dotted line represents a variability plot, showing for each amino acid position how many of 30 hRSV F protein sequences from GenBank were not identical to the A2 sequence. The black lines in the upper part of the figure represent the epitopes or antigenic regions identified by Rock *et al.* [67], Levely *et al.* [71], van Bleek *et al.* [70] or in the present thesis, chapter 3.1 [226] or chapter 3.3.

Does the hRSV G protein contain HLA class I-restricted CTL epitopes?

Stimulation of PBMC with autologous rMVA-F infected cells not only resulted in the identification of several antigenic regions containing Th epitopes as described above, but also three antigenic regions containing HLA class I-restricted CTL epitope(s). In contrast, similar stimulations with rMVA-G infected cells resulted in the identification of G-specific $CD4^+$ T cells only.

HLA genotyping of the individuals used in **chapter 3.3** showed that donor 2 had the HLA-DPB1*0401/0402 genotype and rMVA-G-stimulated bulk cultures generated from this donor all recognized a 15-mer peptide comprising the G-specific epitope described in **chapter 3.2** ($G_{162\text{-}175}$). Although donors 1 and 3 were both HLA-DPB1*0201 positive, the G-specific T cell lines of these two donors did not recognize this peptide. In

almost all cases G-specific responses were directed to the central conserved region of the G protein ($G_{130-230}$). However, this does not exclude that the variable regions of the G protein do not induce Th cell responses. Due to the high variability in the G protein, the G sequences of the viruses that have infected the adult donors during their lifetime were probably relatively distant from that of the A2 virus used to generate the rMVA-G virus. Therefore, if Th responses to the variable regions of the G protein existed in these donors, they were simply not stimulated by the rMVA-G virus.

One of the initial goals of the study described in **chapter 3.3** was to identify possible G-specific $CD8^+$ CTL responses. It has been described that G-specific CTL do not exist in BALB/c mice, nor have they ever been detected in humans [63,64]. rMVA-G infection of APC could lead to presentation of HLA class I-restricted epitopes, and could therefore be used to enrich PBMC bulk cultures for G-specific CTL. However, although in some cultures low G-specific responses were detected, these all proved to be false positive, since the results could not be reproduced and restimulation with rMVA-G-infected APC did not result in outgrowth of specific cells. Since these results were only generated in three individuals, we can not conclude that G-specific CTL are not present in humans. Optimization of the restimulation protocol using rMVA-F infected cells will have to be performed, especially addressing the use of antigen presenting cells (APC) such as monocytes or dendritic cells. Using rMVA-G infected APC in an optimized protocol and a higher number of donors will allow more conclusive statements about the presence of G-specific CTL.

The efficacy and safety of two new candidate hRSV vaccines

Since the vaccination failures in the 1960s, in which a formalin-inactivated alum-formulated hRSV had been used [85], a number of studies have been or are performed on the development of new and safe RSV vaccines [118,119]. Results from animal models of FI-hRSV-mediated enhanced disease suggested that a predominant Th2 response in the absence of a counterbalancing CTL response was one of the factors predisposing for enhanced disease upon hRSV infection [86,126,128]. Therefore, it can be hypothesized that live vaccines or recombinant viral vectors, capable of inducing both class I- and class II-restricted responses, would be better vaccine candidates than inactivated vaccines [131-134,136], but also new generation inactivated vaccines are currently tested [137]. In **chapter 4** the evaluation of the safety and efficacy of two candidate new hRSV vaccines in infant macaques is described: in **chapter 4.1** a subunit vaccine based on the G protein of hRSV [242] and in **chapter 4.2** rMVA mediating the expression of the hRSV F and G genes (rMVA-F/G) [257]. A large number of hRSV vaccination and challenge studies have been carried out by other groups in mice and cotton rats. Since correlates of FI-hRSV-mediated immunopathology have been or are being established in these species, they allow comparison of the immunopathological safety of candidate new

vaccines with FI-hRSV. However, it remains difficult to extrapolate results obtained from SPF inbred rodents to humans. A hRSV vaccination and challenge model in infant cynomolgus macaques was previously developed at Erasmus MC, in which components of FI-hRSV-mediated immunopathology could be reproduced and correlates of enhanced disease could be detected [128]. This model will be useful for preclinical studies with new candidate hRSV vaccines before proceeding to clinical trials in seronegative infants and was used for the evaluation of safety and efficacy of the two new candidate RSV vaccines mentioned above.

Vaccination of infant macaques induced hRSV-specific IgG in 3 of 4 BBG2Na and 4 of 4 rMVA-F/G animals. However, specific IgG responses in all BBG2Na and 2 of 4 rMVA-F/G animals were considered gray zone according to the kit's manufacturer. Levels of vaccine-induced virus neutralizing (VN) antibodies were low in 2 of 4 rMVA-F/G-vaccinated animals and undetectable in the other two rMVA-F/G-vaccinated and all BBG2Na-vaccinated animals. These data are in contrast with results published earlier. Vaccination with BBG2Na induced hRSV-specific IgG and/or neutralizing antibodies in mice, cotton rats and African green monkeys [143,241]. Wyatt *et al.* [156] were able to show high antibody levels in mice vaccinated with a double recombinant rMVA-FG. In addition, Stittelaar *et al.* [229] showed high levels of VN antibodies after vaccination of cynomolgus monkeys with rMVA expressing the measles virus fusion protein (F) and haemagglutinin (H) genes.

Proliferative as well as cytokine responses were also difficult to detect after vaccination with BBG2Na or rMVA-F/G. Vaccination with rMVA-F/G resulted in detectable proliferative responses in only 2 out of 4 animals after vaccination, but cytokine responses were not detected during both the vaccination and challenge period. In BBG2Na-vaccinated animals proliferative responses were also detected in 2 out of 4 animals after vaccination, but these proliferative responses were also accompanied by the production of low levels of IL-13. These results, low antibody levels and low/no proliferative responses, could be explained by the vaccination strategy or vaccination dose especially for rMVA-F/G. Nilsson *et al.* [244] showed that vaccinating three times with rMVA-SIV resulted in high levels of antibodies and proliferative responses in 3 of 4 animals and that vaccinating with rMVA-SIV twice followed by a boost with protein resulted in high levels of antibodies and proliferative responses in all animals. Perhaps a combination of the two hRSV vaccines evaluated in **chapter 4** in a prime-boost vaccination regime could provide a promising vaccination strategy resulting in induction of HLA class I- and class II-restricted T cell responses in combination with high VN antibody levels. In such a scheme, the rMVA-F/G vaccination should be given as prime, while BBG2Na and/or a purified F protein vaccine [138-140] could be considered as boost. However, although such a strategy might result in proof of principle, application in young infants would give substantial practical constraints.

Upon challenge with hRSV no differences in pulmonary hRSV load could be detected between vaccinated and control animals. This could partly be explained by the high challenge dose given to the animals, although with the same dose partial protection was previously detected in FI-hRSV-primed infant macaques [128]. Also, the challenge was given intra-tracheally to ensure delivery of the virus into the lungs, which perhaps renders the model artificial when compared to the natural route of hRSV infection.

Specific antibody responses detected in vaccinated animals after challenge were generally higher and appeared earlier than those in the control animals, indicating that vaccination with both rMVA-F/G and BBG2Na had primed for a secondary immune response. Low levels of eosinophils were detected in broncho-alveolar lavages of 2 of 4 BBG2Na-vaccinated animals and in none of the Rmva-F/G-primed animals.

Conclusions

The results presented in this thesis show that hRSV infection in humans results in a multifaceted immune response, which cannot be described as purely Th1- or Th2-like. However, the observed higher level of IL-13 producing hRSV-specific T cells in infants hospitalized with severe hRSV bronchiolitis could provide a clue for an immunopathological mechanism of natural hRSV-mediated severe disease. Another hRSV-specific immunological factor potentially involved in the pathogenesis of severe hRSV disease could be the frequency and/or phenotype like those of HLA-DP4-restricted T cell responses directed to the conserved region of the RSV G protein. The BBG2Na- and rMVA-F/G-based vaccination strategies evaluated in infant macaques resulted in low VN and cellular immune responses and no detectable protection. A combination of both approaches in a prime-boost regime could possibly increase vaccine immunogenicity, but in this case the immunopathological safety would again have to be evaluated in different animal models.

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Nederlandse Samenvatting

Respiratoir syncytieel virus

Het respiratoir syncytieel virus (RSV) is een veroorzaker van luchtweginfectiesvan de mens. Het komt bij de mens met name in het winterseizoen voor. Infecties met RSV blijven in veel gevallen beperkt tot de bovenste luchtwegen en gaan dan vaak gepaard met milde verkoudheidsverschijnselen. Vooral bij kleine kinderen, personen met een verzwakt afweersysteem of bejaarden kan het virus ook een lagere luchtweginfectie veroorzaken, die gepaard kan gaan met veel ernstiger klinische symptomen. Zo is RSV de belangrijkste veroorzaker van ziekenhuisopnames ten gevolge van ernstige luchtweginfecties bij kinderen onder de zes maanden. Prematuren of kinderen met hartaandoeningen of longproblemen vormen in het bijzonder risicogroepen voor deze ernstige RSV-infecties.

Het menselijk afweersysteem tegen ziekteverwekkers kan worden ingedeeld in twee compartimenten: een niet-specifiek en een specifiek deel. Het niet-specifieke deel is de eerste barrière die ons lichaam moet verdedigen tegen indringers, zoals virussen. Hierbij horen fysieke barrières (onder andere huid, slijmvliezen en maagzuur), afweercellen (onder andere macrofagen en 'natural killer cellen') en moleculen (onder andere interferon, lysozym en complement). Bij herhaalde infecties zal dit niet-specifieke deel van het afweersysteem niet veranderen.

Het specifieke deel van het afweersysteem is in staat om onderscheid te maken tussen 'lichaamseigen' en 'lichaamsvreemde' structuren. Bij infectie met een ziekteverwekker wordt een immunologisch geheugen opgebouwd, waardoor bij herhaalde blootstelling aan dezelfde ziekteverwekker de reactie van het afweersysteem sneller en sterker zal zijn. De belangrijkste cellen van het specifieke afweersysteem zijn twee soorten witte bloedcellen: B-lymfocyten (B-cellen) die, als ze uitgerijpt zijn tot plasmacellen, anti-lichamen produceren en T-lymfocyten (T-cellen) die virussen in geïnfecteerde cellen kunnen bestrijden.

Veel van de 'klassieke' kinderziekten zoals bof, mazelen of rode hond krijgt een mens slechts éénmalig. Bij een herhaalde blootstelling aan deze virussen zorgt het immunologische geheugen voor een snelle reactie van het specifieke afweersysteem, waardoor geen ziekteverschijnselen zullen optreden. Kinderen die zijn gevaccineerd met het zogenaamde BMR-vaccin zullen immuum zijn tegen deze virusinfecties: door de vaccinatie wordt ook immunologisch geheugen opgebouwd, waardoor al bij de eerste blootstelling aan deze virussen de afweerrespons zo snel en sterk is, dat geen ziekte optreedt. Bij RSV is het immunologische geheugen niet in staat om herhaalde infecties te voorkomen, maar dit geheugen biedt wel in toenemende mate bescherming tegen lagere luchtweginfecties. Gedurende het leven kan men

dus herhaaldelijk een RSV-infectie doormaken, die meestal slechts tot verkoudheidsverschijnselen zal leiden.

Een effectief vaccin tegen RSV bestaat nog niet, al doet men hier reeds lang veel onderzoek naar. Dit onderzoek wordt bemoeilijkt door een aantal factoren. Aangezien ernstige infecties vaak op jonge leeftijd voorkomen, zou een vaccin al op zeer jonge leeftijd toegediend moeten worden, een periode waarin het afweersysteem nog niet volledig ontwikkeld is en kinderen nog maternale anti-lichamen hebben. Daarnaast zijn er in de jaren zestig van de vorige eeuw tijdens vaccin-studies ernstige problemen opgetreden. Bij deze studies vaccineerde men jonge kinderen met een experimenteel RSV-vaccin. Tijdens het daaropvolgende RSV-seizoen bleken deze kinderen niet alleen niet beschermd, maar ging infectie met RSV juist met een veel ernstiger ziektebeeld gepaard en zijn er uiteindelijk zelfs twee kinderen overleden. De door het vaccin geïnduceerde afweerrespons bleek in dit geval de boosdoener: als het afweersysteem zelf de ziekte veroorzaakt noemen we dit 'immuunpathologie'. Het exacte mechanisme waarmee het afweersysteem in dit specifieke geval de ziekte veroorzaakte is echter nog niet volledig begrepen, waardoor de angst blijft bestaan dat ook andere RSV-vaccins vergelijkbare problemen zouden kunnen veroorzaken.

Dit proefschrift

Dit proefschrift beschrijft een aantal aspecten van de specifieke afweerrespons tegen RSV-infecties bij kinderen en volwassenen. Daarnaast zijn de veiligheid en effectiviteit van twee kandidaat RSV-vaccins getest in een proefdiermodel. Het onderzoek beschreven in dit proefschrift richtte zich op de beantwoording van een aantal specifieke vragen:

Is de afweerrespons in kinderen met een milde of ernstige RSV-infectie verschillend?

Het onderzoek beschreven in **hoofdstuk 2** van het proefschrift maakte deel uit van het Vigall-project (afkorting van virusgemedieerde allergie), een samenwerkingsproject tussen drie afdelingen van het Erasmus MC. Hoofddoel van dit project was om te kijken of er een verband bestond tussen het doormaken van virale infecties op jonge leeftijd en het ontstaan van allergische ziekten op latere leeftijd. In dit project is een groep kinderen gevolgd tijdens de eerste twee levensjaren en zijn klinische materialen (bloed en cellen van het neusslijmvlies) afgenomen tijdens routineafspraken en na acute luchtweginfecties. Specifieke doelstelling van het in dit proefschrift beschreven deel van het project was om de RSV-specifieke T-cel respons te karakteriseren en vervolgens in verband te brengen met de latere ontwikkeling van allergie. Helaas bleek het aantal gediagnosticeerde RSV-infecties bij deze groep kinderen lager dan verwacht, waardoor het beantwoorden van de oorspronkelijke vraagstelling onmogelijk bleek. De uiteindelijke studie beschrijft het verschil in T-cel respons tussen kinderen met een bovenste luchtweginfectie - al dan niet veroorzaakt door RSV - en

kinderen met een ernstige RSV-geïnduceerde, lagere luchtweginfectie. Hieruit bleek dat het aantal specifieke T-cellen hoger was bij kinderen met een ernstige infectie dan kinderen met een milde infectie, maar nauwelijks verschilde tussen de twee groepen met milde infecties. Tevens bleek het aantal T-cellen dat het cytokine interleukine (IL)-13 produceerde, hoger te zijn bij kinderen met ernstige dan bij kinderen met milde infecties. Dit cytokine zou mogelijk een verband kunnen vormen tussen RSV op jonge leeftijd en het ontstaan van allergische ziekten op latere leeftijd, aangezien IL-13 een centrale rol speelt bij het ontstaan van astma. Om dit nader te kunnen bestuderen zullen grotere prospectieve cohortstudies nodig zijn.

Hoe ziet de RSV-specifieke T-cel respons eruit?

In **hoofdstuk 3** worden twee verschillende manieren beschreven om te kijken naar de RSV-specifieke T-cel respons. In **paragraaf 3.1** zijn cytotoxische T-cel kloons geïsoleerd uit het bloed van twee kinderen na een RSV-infectie. De T-cel kloons herkenden verschillende eiwitten van RSV: het fusie-eiwit (F) en de 1B- en 1C-eiwitten. De F-specifieke kloons werden nader bestudeerd en herkenden minimale epitopen van negen aminozuren in de context van HLA-B*57 of -C*12.

In **paragraaf 3.2** zijn T-helpercel kloons geïsoleerd uit neusslijmvlies en bloed van twee andere kinderen. Eén kloon was gericht tegen het matrix-eiwit van RSV en herkende het minimale epitoop in de context van HLA-DPB1*1601, de andere was specifiek voor het aanhechtingseiwit (G) van RSV en herkende het minimale epitoop in de context van HLA-DPB1*0401 en -0402. Interessant genoeg bleek het minimale epitoop van de laatste T-cel kloon te liggen in het geconserveerde gedeelte van het G-eiwit. Omdat het restrictie-element HLA-DP4 wereldwijd vaak voorkomt, zou een groot deel van de wereldbevolking dit epitoop na iedere RSV-infectie kunnen herkennen. Studies in proefdiermodellen suggereren dat de afweerrespons tegen G mogelijk een belangrijke rol speelt in de uitkomst van de RSV-infectie, daarom is gekeken of de respons tegen dit epitoop een beslissende factor zou kunnen zijn bij het ontwikkelen van ernstige ziekte na RSV-infectie. Hiervoor zijn cellijnen van kinderen met een milde of ernstige, lagere luchtweginfectie gebruikt om het minimale epitoop te presenteren aan de G-specifieke T-cel kloon. Uit de resultaten bleek echter dat de mogelijkheid om dit epitoop te presenteren geen aantoonbare invloed heeft op de uitkomst van de infectie.

Een andere manier om naar de RSV-specifieke afweerrespons te kijken is beschreven in **paragraaf 3.3**. Hiervoor zijn T-cellen van drie gezonde volwassen donoren gestimuleerd met een recombinant gemodificeerd vaccinia virus Ankara (rMVA) dat zorgt voor de expressie van het F- (rMVA-F) of G- (rMVA-G) eiwit van RSV. Hierdoor zullen de eiwitspecifieke T-cellen uitgroeien en kan bepaald worden tegen welke gedeelte van het eiwit deze gericht zijn. Na stimulatie met rMVA-F is een set overlappende peptiden gebruikt om te kijken tegen welk gedeelte van het F-eiwit deze T-cellen gericht waren. Uit de resultaten van deze studie bleek, dat

deze cellen verschillende regio's van het F-eiwit herkenden. In combinatie met een andere studie bleek zelfs dat verspreid over het gehele F-eiwit regio's voorkomen, die herkend kunnen worden door T-cellen. Na stimulatie met rMVA-G bleek dat bijna alle specifiek uitgroeiende T-cellen gericht waren tegen het constante gedeelte van het G-eiwit. Tevens waren alle G-specifieke cellen van het CD4-positieve T-helper fenotype, waarmee een andere vraag ook beantwoord kon worden: in tegenstelling tot het F-eiwit lijkt het G-eiwit geen CD8-positieve cytotoxische T-cellen te stimuleren. Om deze vraag definitief te kunnen beantwoorden moet nog wel een groter aantal donoren getest worden. Hiervoor lijkt de in deze paragraaf beschreven methode zeer geschikt.

Twee kandidaatvaccins tegen RSV: zijn ze veilig en effectief?

In **hoofdstuk 4** zijn twee kandidaatvaccins geëvalueerd met betrekking tot hun veiligheid (veroorzaken ze immuunpathologie na infectie met RSV?) en effectiviteit (beschermen ze tegen infectie met RSV?). Deze studies zijn uitgevoerd in een apenproefdiermodel, waarin eerder de immuunpathologie veroorzaakt door het in de jaren zestig gebruikte vaccin ('FI-RSV') kon worden gereproduceerd.

In **paragraaf 4.1** werd een subunitvaccin getest, gebaseerd op het G-eiwit van RSV (BBG2Na) en in **paragraaf 4.2** een rMVA dat het F- en G-eiwit van RSV (rMVA-F/G) tot expressie brengt. De vaccinatie met deze twee kandidaatvaccins is getest in vergelijking met FI-RSV-vaccinatie. Na vaccinatie met de twee vaccins konden geen T-cel responsen en slecht in een aantal dieren RSV-specifieke anti-stoffen aangetoond worden. Wel werden IL-13 producerende T-cellen aangetoond na vaccinatie met BBG2Na (bij twee van de vier apen) en FI-RSV (bij beide apen), hoewel deze respons in de BBG2Na-gevaccineerde apen beduidend lager was dan in de FI-RSV-gevaccineerde apen. In een eerdere studie met FI-RSV bij makaken werd productie van IL-13 geassocieerd met een ernstiger ziektebeeld. Na experimentele infectie van de dieren met RSV kwamen RSV-specifieke (neutraliserende) anti-stoffen snel op, wat betekent dat de apen wel immunologisch geheugen hadden opgebouwd tegen RSV. Helaas bleek deze respons niet te leiden tot een aantoonbare bescherming tegen de RSV-infectie: de hoeveelheid virus in gevaccineerde dieren was vergelijkbaar met die in niet-gevaccineerde dieren. In twee van de vier BBG2Na- en beide FI-RSV-gevaccineerde apen werden na RSV-infectie in de longen eosinofiele granulocyten aangetoond, cellen die samenhangen met een allergische reactie. Het ging hierbij om dezelfde dieren waarbij ook IL-13 productie was gezien, maar ook hier waren de waarden lager in de BBG2Na-gevaccineerde apen dan bij de dieren gevaccineerd met FI-RSV. Uit deze studies is gebleken dat beide nieuwe vaccins niet genoeg bescherming boden en dat gezocht moet worden naar betere en veiligere vaccins of andere vaccinatiestrategieën.

Kortom: in dit proefschrift zijn studies beschreven, waarbij de afweerrespons tegen RSV-infecties is bestudeerd in relatie tot het vóórkomen of juist voorkómen van ziekte. De resultaten van deze studies kunnen een bijdrage leveren aan een beter begrip van de processen waarmee RSV-infecties ziekte veroorzaken en aan de verdere ontwikkeling van een RSV-vaccin.

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Dus samengevat: BEDANKT ALLEMAAL!

Curriculum Vitae

De schrijver van dit proefschrift werd op 9 juli 1975 geboren te Rotterdam. Na de kleuterschool en de lagere school in deze plaats doorlopen te hebben, werd in 1993 het gymnasium-β-diploma behaald aan de RKSG Sint-Montfort (het huidige NOVA college-Montfort, eveneens te Rotterdam). In 1993 begon hij aan de opleiding Biomedische Wetenschappen aan de Universiteit van Leiden. Tijdens deze studie heeft hij stages uitgevoerd aan de afdelingen Anthropogenetica en Tumorimmunologie van het Leids Universitair Medisch Centrum en de afdeling Urologie van het Erasmus Medisch Centrum te Rotterdam. In 1998 rondde hij de betreffende universitaire opleiding af, waarna hij in juli 1998 assistent in opleiding op de afdeling Virologie van het Erasmus MC werd. Onder de begeleiding van prof.dr. A.D.M.E. Osterhaus en dr. R.L. de Swart werd het promotieonderzoek uitgevoerd, dat heeft geleid tot de totstandkoming van dit proefschrift.

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