Evasion of Influenza A Viruses from Human T-cell Immunity

Het ontsnappen van influenza A virussen aan humane T-cel immuniteit

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 28 februari 2007 om 11.45 uur

door

Eufemia Gabriella Maria Berkhoff
geboren te Delft

Promotor:
Prof.dr. A.D.M.E. Osterhaus

Overige leden:
Prof.dr. E.H.J.H.M. Claassen
Prof.dr. J.J.M. van Dongen
Prof.dr. E.J.H.J. Wiertz

Co-promotor:
Dr. G.F. Rimmelzwaan

ISBN: 9056773062

The studies presented in this thesis were conducted at the Department of Virology of the Erasmus Medical Center in Rotterdam, The Netherlands, with financial support from the European Union (Grant QLRT-2001-01034: Novaflu). The promotion was financially supported by Solvay Pharmaceuticals BV, Roche Nederland BV, ViroClinics BV, BD Biosciences, ViroNovative BV and Greiner Bio-One BV.

Cover illustration: Electron micrograph of a T-cell recognizing an antigen presenting cell (courtesy of Lynn Yarris).

Production: PrintPartners Ipskamp, Rotterdam, The Netherlands

Voor mijn papa.

List of abbreviations

A	alanine
Ab	antibody
ANOVA	analysis of variance
APC	antigen presenting cell
APC	allophycocyanin
BLCL	B-lymphoblastoid cell line
C	cysteine
C1R	B-cell negative for HLA-A and -B expression
CD	cluster of differentiation
CTL	cytotoxic T-lymphocyte
D	aspartic
DNA	deoxyribonucleic acid
E	glutamic
EBV	Epstein-Barr virus
ELISPOT	enzyme linked immunospot
ER	endoplasmatic reticulum
E : T	effector-to-target
F	phenylalanine
FACS	fluorescence activated cell scanner
FATT-CTL	fluorescent antigen-transfected target cell - cytotoxic T-lymphocyte
FITC	fluorescein isothiocyanate
G	glycine
GFP	green fluorescent protein
H	histidine
HA	hemagglutinin
HI	hemagglutinin inhibition
HIV	human immunodeficiency virus
HK	Hong Kong
HLA	human leukocyte antigen
HPLC	high pressure liquid chromatography
I	isoleucine
ICS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ISD	influenza sequence database
IU	international units
K	lysine
L	leucine
M	methionine
M1/M2	matrix protein
MAb	monoclonal antibody

MDCK	Madin-Darby canine kidney
MDS	multi-dimensional scaling
MHC	major histocompatibility complex
MOI	multiplicity of infection
MT	mutant
N	asparagine
NA	neuraminidase
NK	natural killer
NL	The Netherlands
NP	nucleoprotein
NS	non-structural protein
P	proline
PB1/PB2/PA	polymerase proteins
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridin chlorophyll protein
PHA	phytohemagglutinin
p.i.	post infection
PR	Puerto Rico
p.t.	post transfection
Q	glutamine
R	arginine
R0.1B	RPMI 1640 supplemented with 0.1% bovine serum albumin and antibiotics
R10F	RPMI 1640 supplemented with 10% fetal calf/bovine serum and antibiotics
R10H	RPMI 1640 supplemented with 10% human pooled serum and antibiotics
RNA	ribonucleic acid
RPMI	culture medium
RT-PCR	reverse transcriptase-polymerase chain reaction
S	serine
SD	standard deviation
SIV	simian immunodeficiency virus
T	threonine
TAP	transporter associated with antigen processing
$TCID_{50}$	tissue culture-infectious dose 50%
TCR	T-cell receptor
Th	T-helper
TNF	tumor necrosis factor
V	valine
W	tryptophan
WHO	World Health Organization
WT	wild type
www	world wide web
Y	tyrosine

Contents

Chapter

1

Introduction and outline of the thesis

Section I: *influenza viruses*

Influenza virus structure

Influenza viruses belong to the family of *Orthomyxoviridae* (52), and are enveloped, single-stranded negative-sense RNA viruses with a segmented genome (145). Influenza virus particles have a spherical shape, and their size ranges from 80 to 120nm in diameter (118, 119). There are three types of influenza virus (A, B, and C), which can be distinguished on the basis of antigenic characteristics of the internal proteins (145). The eight RNA-segments of influenza A and B viruses and seven segments of influenza C viruses are independently encapsidated by the viral nucleoprotein (NP) and associated with polymerase proteins PB2, PB1 and PA, which forms the ribonucleoprotein (RNP) complex (135, 181). The seven or eight RNP complexes are located inside a shell of matrix 1 protein (M1) (86), which lines the viral envelope (figure 1). The envelope of influenza viruses is derived from the plasma membrane of the host cell in which the virus has replicated (130). Embedded in the viral envelope is the matrix 2 protein (M2), which forms membrane ion-channels (43, 146, 193). Influenza A and B viruses have two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), which form a typical layer of spikes radiating outward from the lipid envelope (149). Influenza C virus exhibits a single multi-functional glycoprotein (145). The HA and NA of influenza A viruses exhibit much greater amino acid sequence variability than those of influenza B viruses, and based on the antigenic differences of their HA and NA, the influenza A viruses are divided into subtypes. To date, 16 HA and 9 NA subtypes have been described for influenza A virus (80, 111, 132, 215, 273). All subtypes, in different combinations, have been isolated from birds, but only 3 HA and 2 NA subtypes have caused recurrent infections in humans (113, 261, 274).

Influenza A virus infection and replication

In the initial phase of influenza virus infection (figure 2), the HA on the influenza virus envelope binds to sialic acid-containing receptors on cells of the host (265, 268). Upon binding, the virus particle becomes endocytosed by the cell and internalized. Human influenza A viruses preferentially bind sialic acid residues attached to galactose by an α2,6 linkage, whereas avian influenza viruses prefer sialic acid attached by an α2,3 linkage (50, 214, 225, 251). The low pH in the endosome causes an acidification of the interior of the virus as H^+ flows through the M2 ion-channels resulting in a dissociation of the M1 from the RNP, allowing the release of RNP (107, 162). The acid pH also triggers a structural change in the HA, which induces fusion of the viral envelope with the membrane of the endosome, resulting in the uncoating and entry of the virus RNP into the cytoplasm (53, 226). The RNP are transported to the nucleus of the cell with interaction of NP (164). The transcription of the viral genome into mRNA is catalyzed by PB1 (68, 117). The PB2 cleaves 5'-capped

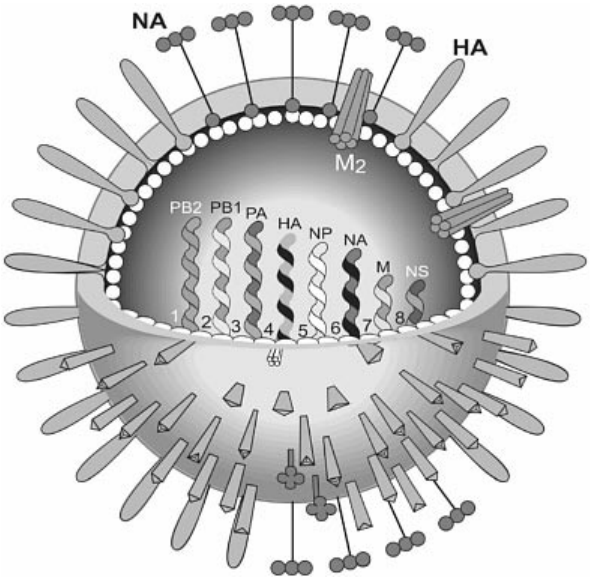


Figure 1. Schematic representation of an influenza A virus particle. Adapted from Smolinski *et al*., Microbial Threats to Health: The Threat of Pandemic Influenza (ISBN: 0-309-09717-7).

fragments from newly synthesized host cell mRNA to provide the primers of viral mRNA synthesis (23, 141, 198). In addition to the 5'-cap, a tail of polyA is added to the transcribed viral mRNA. The mRNA is transported back to the cytoplasm and translated into protein (224). The newly synthesized HA, NA, and M2 proteins are transported through the endoplasmic reticulum (ER) and Golgi apparatus to the plasma membrane of the cell (177, 228). The newly synthesized PB2, PB1, PA and NP re-enter the nucleus to form new RNP (177, 228). The nonstructural 1 protein (NS1) inhibits pre-mRNA splicing, polyadenylation and nuclear export of host mRNA (204). This sequesters host mRNA in the nucleus that not only supplies a source of cap structures for viral transcription but also inhibits host gene expression. In addition, NS1 protects viral replication from host immune responses (15, 77, 235, 258). To switch from transcription to replication, complementary positive-stranded intermediate RNA (cRNA) is initiated without priming, representing a full-length copy of the vRNA (106). The cRNA then serves as a template for the synthesis of negative-stranded progeny vRNA. The PA is required for both transcription and replication (104, 175). To end replication, M1 enters the nucleus and binds to the RNP. The M1 and nonstructural 2 protein (NS2) mediate the nuclear export of the new formed RNP (164). A complex of RNP, M1 and NS2 buds from the apical side of the plasma membrane, which contains the viral HA, NA and M2 proteins, resulting in the assembly of new virus particles (177, 228, 279). To prevent clumping of released virus due to HA binding, the NA cleaves the sialic acid from the virion glycoproteins and the cell (177). Extracellularly, the HA is cleaved into two subunits (HA1 and HA2) which is a prerequisite for infectivity, since the amino terminus of HA2 functions

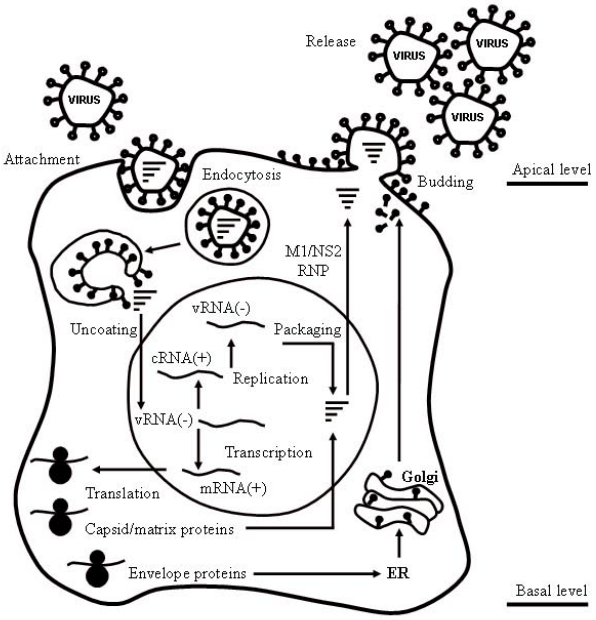


Figure 2. Schematic representation of the replication cycle of influenza A virus. Redrawn from Lamb *et al.*, Orthomyxoviridae: the viruses and their replication. In D. M. Knipe, P.M. Howley, D.E. Griffin (ed.), Fields Virology (145).

to fuse the viral envelope with the endosomal membrane on uncoating as described earlier (226). The enzyme responsible for cleavage is a trypsin-like protease, which is released from cells in the respiratory epithelium. Due to the limited distribution of this enzyme, influenza virus infection is normally restricted to the respiratory tract. Progeny virus can infect other cells or can be transmitted to new individuals. The entire process of viral infection seriously disrupts the normal physiology of the cell, eventually leading to cell death. However, the cytopathic effect does not occur until the cell has produced many thousands of new virus particles.

Influenza virus pathogenesis

Influenza viruses cause acute respiratory infections, are highly contagious and have afflicted humans and animals since ancient times with significant morbidity and mortality on an epidemic and pandemic scale. In 1918, influenza virus H1N1 caused over twenty million deaths worldwide and probably ten times more people were affected, which is referred to as the '*Spanish flu*' pandemic (236, 260). At present, epidemics occur throughout the world in the human population due to infection with influenza A viruses of subtypes H1N1 and H3N2 or with influenza B virus. Influenza C virus only causes mild disease. Annually, in-

fluenza virus causes 3 to 5 million cases of severe illness and is fatal to 0.25 to 0.5 million people worldwide. Especially young children, the elderly, pregnant women, individuals with chronic cardiopulmonary conditions and the immunocompromised are at risk (269). Since influenza A viruses are responsible for all pandemic and most epidemic outbreaks, comments in this thesis are restricted to influenza A virus, unless otherwise stated.

Since 1996, influenza viruses H9N2, H5N1 and H7N7 have been transmitted from birds to humans (47, 81, 136, 142, 187, 233, 286), but have failed to spread in the human population on a large scale. Although, more than 250 confirmed cases of influenza virus H5N1 infection, of which more than 150 fatal, have been reported to the World Health Organization (WHO) since December of 2003 (272), and further global spread of these viruses is feared. Surveillance studies suggest that migratory birds and waterfowl are the source of all influenza A viruses in nature, but influenza A viruses can infect a large variety of animal species, including pigs, horses, sea mammals, felines and dogs (112, 274).

Section II: *the immune system*

The immune system protects the host from foreign substances by exploiting a variety of immune responses. The innate response is regarded as predetermined, and is directed towards a broad range of pathogens. The adaptive response is regarded as acquired, and relies on specific recognition and reactivity. The adaptive response can be divided into a humoral response and a cellular response. However, there is no clear demarcation between different immune responses, as the different responses coexist, co-function and complement each other (1, 59, 60, 110).

The innate response

Innate immunity provides the early lines of defense against pathogens, including influenza viruses. The presence of nucleic acids not typically associated with host cell replication or protein synthesis can be recognized by infected epithelial cells, monocytes, macrophages and dendritic cells, initiating signals that lead to the secretion of cytokines (2). Type I interferons (IFN-α and IFN-β) are among the most important cytokines produced in the innate response, as they are highly effective in early suppression of viral replication at the transcription-translation level (238), however, activation of type I interferons may be inhibited by influenza A virus NS1 (15, 77, 235, 258). Furthermore, cytokines attract macrophages, neutrophils, dendritic cells and natural killer (NK) cells to the site of infection, which produce more cytokines, facilitating activation of the adaptive response, and enhancing antigen presentation and maturation of antigen presenting cells (150). Cells present their contents to the immune system to indicate if they are infected or not via major histocompatibility complex (MHC) class I molecules, which are highly polymorphic cell-surface glycoproteins

that present peptides derived from cellular or viral proteins (in humans, MHC-molecules are often referred to as human leukocyte antigen (HLA) molecules followed by a subtype indication) (1, 7-9). In the innate response, MHC class I-presentation is important for NK cell regulation, since recognition of MHC class I complexed with peptides derived from self proteins triggers an inhibitory signal to the NK cell. The absence of MHC class I on the cell surface or presentation of non-self peptides abolishes the inhibitory signal, leading to elimination of the cell by the release of perforins (perforates the cell membrane) and granzymes (induces cell apoptosis) (108, 147, 157, 230). Type I interferons enhance the ability of NK cells to eliminate infected target cells (69). A second NK cell population, the NK-T cells, consists of unconventional T-cells that are restricted by a non-polymorphic molecule, CD1d, which presents self and foreign lipid antigens (22, 33, 152). NK-T cells can lyse infected cells via Fas/Fas ligand (FasL) interactions and secrete copious amounts of various cytokines, such as interleukin-4 (IL-4) and gamma interferon (IFN-γ). IFN-γ induces synthesis of proteins that inhibit viral replication by impairing accumulation of virus-specific mRNA, dsRNA and proteins (188), stimulates antiviral immune responses by promoting MHC-presentation on antigen presenting cells (76), augments the proteolysis and peptide transport in antigen presenting cells (275), activates immune cells (78, 161), and serves as a regulator of the innate and adaptive response. Another subset of proteins important in the innate response is the complement system, which is comprised of a group of serum proteins that can act in an enzymatic cascade, providing a number of molecules involved in inflammation, cell lysis and phagocytosis (1, 256, 257). The complement components C5b, C6, C7, C8 and C9 form a membrane-attack complex that perforates cell membranes, which leads to cell death, and the cleavage fragment of complement component C3, the C3b molecule, enhances phagocytosis by macrophages and neutrophils (90, 121, 252). Macrophages present the phagocytosed pathogens to antigen-specific T-lymphocytes (139, 244, 281). Also dendritic cells constantly endocytose extracellular antigens, and become activated and behave as antigen presenting cells when their pattern-recognition receptors, including toll-like receptors, recognize distinctive pathogen-associated molecular patterns on the surface of microorganisms (3, 131). The release of IFN-α from virus-infected cells also activates dendritic cells (238). Dendritic cells are extremely efficient at presenting antigens to $CD4^+$ T- helper cells and $CD8^+$ T-cells (see below), and are the principal antigen presenting cells for activating naïve T-cells (4, 82, 127).

The adaptive response

The adaptive response is characterized by the high specificity for distinct molecules, and the ability to remember and respond more vigorously to repeated exposures of the same pathogen. The adaptive response is classified into a humoral antibody-mediated response and a cellular T-lymphocyte-mediated response. Neutralizing antibodies are secreted by B-lymphocytes, and bind to virus particles and infected cells that present virus proteins on

their membranes, blocking virus-entry into host cells and promoting phagocytosis by macrophages (39, 174, 278). Antibody isotype IgA is locally secreted by mucosal epithelia, and plays a critical role in the defense against respiratory pathogens, like influenza virus (48, 206). In addition, IgA and antibody isotype IgG are secreted systemically, and can persist in serum for years (36, 173). Virus-infected cells, which are coated with IgG antibody, are also recognized and lysed by NK cells. Pre-existing virus-specific antibodies induced by prior infection or vaccination can provide immediate neutralizing immunity to subsequent influenza virus infections.

T-lymphocytes can be identified by their CD3 receptor, and can be divided into two functionally distinct populations, the $CD8^+$ cytotoxic T-lymphocytes (CTL) and the $CD4^+$ T-helper cells. Upon recognition of antigen presented by dendritic cells (4), naïve $CD8^+$ T-lymphocytes differentiate and expand clonally into mature activated effector cells, and migrate from the lymph nodes or spleen into the blood circulation and intercellular fluids. The $CD8^+$ T-lymphocytes act via secretory and cell contact-dependent pathways by T-cell receptor (TCR) contact. The TCR conjointly with the CD8 and CD3 receptor recognizes infected cells via foreign peptides presented by the MHC class I-molecule. During virus replication in the cell, viral proteins are captured by the proteosome, which cleaves proteins into smaller peptides. The transporter associated with antigen processing (TAP) transports the peptides into the ER, where the peptides are further processed (8-11 amino acids in length) and bound to MHC class I-molecules. The MHC-peptide complex is transported through the Golgi apparatus to the cell surface, where it is presented as epitope to CTL (figure 3). Upon recognition of the epitope, CTL eliminate the infected cells directly and rapidly by granule exocytosis of perforins and granzymes (128, 247). Alternatively, CTL can eliminate cells via Fas/FasL or tumor necrosis factor (TNF) receptor 1 interactions, which also trigger apoptotic death of target cells (85, 242). The secretory pathway involves release of inflammatory cytokines, including IFN-γ and TNF-α, to exert paracrine effects on cells in the vicinity, promoting MHC class I-expression and entry into an antiviral state. Although virus-specific CTL cannot prevent infection of host cells, like virus-neutralizing antibodies, they promote viral clearance and accelerate recovery from infection by eliminating virus-infected cells, limiting the production of progeny virus (12, 165, 166, 168).

The $CD4^+$ T-helper cells have an immune response promoting and regulating function, and can be divided into type 1 and 2 cells. T-helper 1 cells facilitate proliferation and differentiation of CTL by secretion of cytokines IL-2, IL-12, IL-15, TNF-α and IFN-α, β, and γ. In humans, T-helper 1 responses also stimulate IgG1 antibody production. IgG1 antibodies can activate the complement system and enhance phagocytosis by macrophages. T-helper 2 cells facilitate proliferation and differentiation of B-lymphocytes to antibody-producing plasma cells by secretion of cytokines IL-4, IL-5, IL-6, IL-10, IL-13, and IL-16 (144). T-helper 2 responses stimulate IgG4 antibody production. In contrast to IgG1, IgG4 antibodies do not activate the complement enzyme cascade. Influenza virus infection gen-

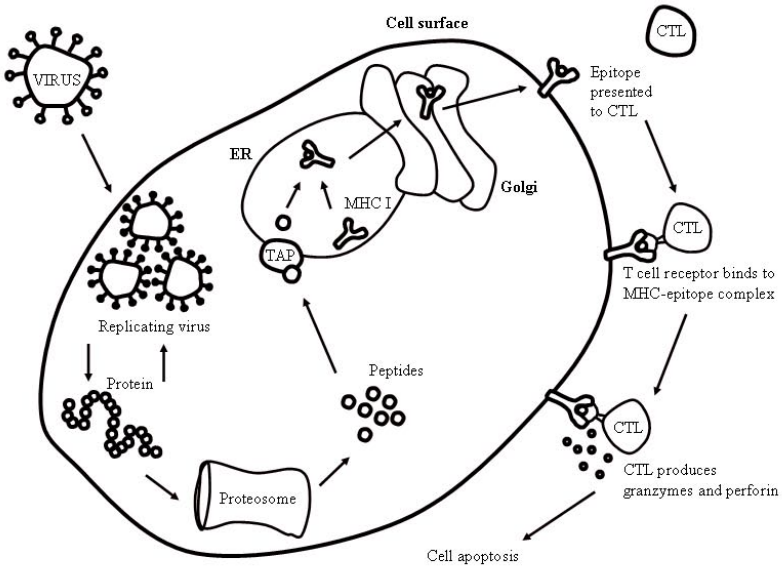


Figure 3. Schematic representation of endogenous antigen processing and MHC class I-presentation. Redrawn from Oldstone, How viruses escape from cytotoxic T-lymphocytes: molecular parameters and players (183).

erally induces a T-helper 1 type response (217, 243). T-helper cells recognize peptides of 10-30 amino acids in length with an exogenous origin, which are bound to MHC class II-molecules (32). A third subset of $CD4^+$ T-lymphocytes constitutes the regulatory T-cells, which are $CD4^+$ $CD25^+$. Regulatory T-cells control the properties of other immune cells by suppressing their functional activity to prevent auto-immunity (40, 182, 285). Whether regulatory T-cells play a role in influenza virus infection is yet unclear (38).

As mentioned above, virus-specific T-lymphocytes expand upon primary antigen recognition, and acquire effector function and change their migratory capacity by expression of co-receptors in accordance with the amount of signal received by the antigen presenting cell and ancillary cytokine stimuli (148). After the virus is cleared, the levels of antigen decline and most virus-specific effector T-cells undergo apoptosis, except for a minor fraction that persist as long-term memory T-cells that divide slowly in the absence of antigen. The survival of these long-term memory T-cells depends on a number of intrinsic properties, like anti-apoptotic molecules, and the expression of cytokine receptors, like those for IL-7 or IL-15 for intermediate and long-term maintenance respectively (89, 148). The studies presented in this thesis are based on memory responses, since peripheral blood mononuclear cells were obtained from study subjects that were exposed once or more to influenza A virus in the past.

Section III: *immune evasion by influenza A virus*

To evade host immune responses, viruses can exploit a variety of mechanisms preventing recognition and elimination. Viruses can impair the humoral immune response by evading antibody neutralization, and the cellular immune response by viral latency, replication in immune-privileged sites, induction of immunosuppresion, induction of CTL clonal exhaustion, interference with proteolysis, TAP-transport, MHC class I- and class II-presentation, by induction of unresponsiveness in dendritic cells, by deceiving NK cells, by inducing amino acid variation in T-cell epitopes, by altered expression of specific viral antigens, and down-regulating adhesion molecules, CD4-expression or T-cell receptors. In addition, viruses can impair cytokine and complement function, cellular signaling and induction of apoptosis. The persistent escape from the immune system may lead to chronic infection. Among the most sophisticated viruses in immune evasion are the pox viruses (218, 220, 229) and the herpes viruses (6, 197, 255), but also other viruses, like lymphocytic choriomeningitis virus (171, 195), adenovirus (13, 91, 219, 241, 270), human immunodeficiency virus (29, 96, 170, 192, 223), hepatitis B virus (21, 79) and hepatitis C virus (100), use one or more of the above strategies to ensure their existence in the presence of the host's immune response. So do the influenza A viruses.

During viral replication, influenza virus NS1 binds the viral dsRNA preventing activation of mediators of the innate immune response, such as cellular protein kinases (15) and interferon regulatory factors (235). Activated protein kinases can block both host and viral protein synthesis, and thus can inhibit viral replication (15). In addition, protein kinases can activate other factors important for antiviral cellular signaling, like NF-κB, which in turn activates antiviral cellular factors like IFN-α and -β, IL-2 and MHC class I-expression (258). Interferon regulatory factors induce the transcription of specific antiviral genes, like IFN-β (235). Influenza virus NS1 also affects the synthesis of chemokine co-receptor 7 (CCR7) and macrophage inflammatory protein 1β (MIP1β) (77), which are important for migration of immune cells (221). Inhibition of transcriptional factors associated with dendritic cell maturation (e.g. NF-κB and AP1) and migration (e.g. MIP1β and CCR7) by influenza virus NS1, diminishes not only the migratory capacity of dendritic cells, but also the optimal stimulation of T-cells, affecting both innate and adaptive responses (77).

However, most notorious are influenza viruses for their escape from neutralizing antibodies directed against the surface glycoproteins HA and NA, which are induced by prior infection or vaccination (92, 232, 262, 284). Unlike DNA replication, there are no proof-reading mechanisms involved in RNA replication, rendering the replication of RNA an error-prone process. As a result, progeny virus incorporate mutations that can accumulate in antigenic sites recognized by antibodies. Gradual antigenic changes in the HA or NA of the prevalent virus, known as antigenic drift, result in the evasion from neutralizing antibodies that exert selective pressure. Antigenic drift variant strains cause the annual

influenza A virus epidemics (145, 274) and necessitates the regular update of the influenza virus vaccine. The introduction of a new HA or NA subtype into the human population is known as antigenic shift (145, 274), and is the result of genetic reassortment between avian and mammalian influenza A viruses, infecting the same host (261). The introduction of a new subtype of influenza A virus into the human population has occurred in 1957, when the H2N2 subtype replaced the H1N1 subtype, and in 1968, when the H3N2 subtype replaced the H2N2 subtype, both resulting in an influenza virus pandemic (46, 57, 145). The introduction of a new HA or NA subtype can also occur upon direct transfer of whole influenza A virus from another species (47, 81, 136, 142, 187, 233, 286), or through the re-emergence of influenza A virus, which may have caused an epidemic many years earlier, like in 1977, when the H1N1 subtype reappeared (176). Currently, avian influenza virus H5N1 poses a pandemic threat, since the H5N1 subtype has not circulated in the human population for at least a century, rendering the human population immunologically naïve to this subtype. Furthermore, the virus may further adapt to humans by gradual mutations or by reassortment with human influenza virus strains, enabling human-to-human transmissions.

The internal proteins of influenza A virus are more conserved than the surface glycoproteins HA and NA. These proteins are less accessible for antibodies, and are targeted by influenza virus-specific $CD8^+$ T-cells. Amino acid mutations in or adjacent to T-cell epitopes do occur, and can affect peptide processing and presentation, binding to MHC class I- and II-molecules, and TCR interaction, and as a result can prevent recognition by virus-specific T-cells (figure 4) (138, 183, 197). This escape mechanism has been described predominantly for chronic virus infections, but it has also been described for influenza A virus (26, 185, 202, 253). The amino acid arginine (R) at position 384 of the NP is an anchor residue for the HLA-B*0801-restricted epitope $NP_{380–388}$ (ELRS<u>R</u>YWAI) and the HLA-B*2705-restricted epitope $NP_{383–391}$ (S<u>R</u>YWAIRTR). An amino acid substitution at this position (R384G) resulted in the loss of the anchor residue and, as a consequence abolished recognition by CTL (253). In the HLA-B*3501-restricted epitope $NP_{418–426}$ variation was observed in the TCR contact residues. These epitope variants emerged over time, and CTL specific for older variants failed to recognize the mutated versions of the same epitope (26).

The primary and single, most cost-effective method of preventing influenza virus infection is vaccination (269). Vaccination results in significant reductions in influenza virus-induced respiratory illnesses, hospitalizations and deaths. In addition, current inactivated influenza virus vaccines have an excellent safety record. But to be effective, the vaccine needs to match the influenza virus that causes the illness, which is a challenge considering antigenic drift and shift. Twice a year, once for the northern and once for the southern hemisphere, the WHO recommends which two influenza A virus strains (H1N1 and H3N2) and which influenza B virus strain should be included in the vaccine. Occasional vaccine mismatches occur and result in reduced efficacy of the vaccine. Another limitation of the

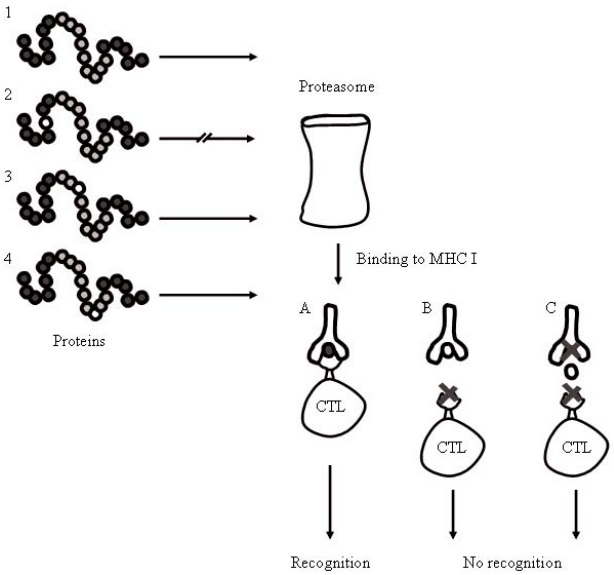


Figure 4. Schematic representation of the effect of a single amino acid substitution on antigen processing, MHC class I-binding and T-cell receptor recognition. The effect of a single amino acid mutation (open circles) in the nine amino acids long epitope (gray circles) or in the sequence flanking the epitope (black circles) on MHC class I-binding and T-cell receptor recognition is depicted. In row 1, the polypeptide is degraded appropriately in the proteosome and translocated by TAP to the ER, where it binds tightly to the MHC class I-molecule. The peptide-MHC complex is then recognized by CTL (A). In row 2, a mutation in the sequence flanking the epitope prevents processing by the proteosome, abrogating presentation of the epitope to CTL. In row 3, a mutation at a TCR contact residue prevents recognition by CTL (B). In row 4, a mutation at an anchor residue abrogates binding to MHC class I, and as a result prevents recognition by CTL (C). This figure was redrawn from Koup, Virus escape from CTL recognition (138).

vaccine is the low efficacy in the elderly, which is related to waning immune responses with age. Collectively, the limitations of the current vaccine call for novel developments in the control of influenza virus. Targets for CTL responses, like influenza virus NP and M1/M2, are considered as candidate vaccines, because of their conserved nature. The use of conserved proteins could provide protective immunity against drift variants or viruses with novel subtypes (246, 283).

Section IV: *outline of the thesis*

Cellular immunity plays an important role in the control of viral infections, including those caused by influenza viruses. However, viruses can exploit a variety of strategies to evade cellular immunity, like the accumulation of amino acid substitutions in CTL epitopes. It was unclear to what extent these amino acid substitutions affect the influenza virus-specific CTL response. In this thesis, this issue was addressed by assessing the effect of the loss of immunodominant epitopes on the human influenza A virus-specific CTL response *in vitro*. To this end, recombinant influenza viruses with and without the HLA-B*2705-restricted epitope $NP_{383-391}$ or the HLA-B*3501-restricted epitope $NP_{418-426}$ were generated, which were used to induce IFN-γ production or lytic activity in clonal and polyclonal virus-specific $CD8^+$ T-cell populations (chapter 2 and 7). During this study, it was found that recombinant influenza viruses with a single amino acid substitution at position 384 of the NP (R384G) could not be rescued. We hypothesized that one or more co-mutations were required to compensate for the detrimental effect of the R384G mutation, which is described in chapters 3 and 4. In line with these results, we hypothesized that influenza A viruses need to overcome functional constraints to accumulate mutations in CTL epitopes and escape from CTL. The inability to overcome these functional constraints may explain the highly conserved nature of most identified influenza A virus CTL epitopes, limiting escape from CTL. To assess the impact of amino acid substitutions in conserved epitopes on viral fitness and recognition by specific CTL, we performed a mutational analysis of CTL epitopes (chapter 5).

Since examples of evading mutations in influenza A virus CTL epitopes are limited, we assessed the extent of variation in CTL epitopes using virus-specific $CD8^+$ T-cell clones (chapter 6). In addition to $CD8^+$ T-cell clones, we also observed $CD4^+$ T-cell clones that recognized a variable epitope. To date, no evading activities from $CD4^+$ T-cells have been described in influenza virus infection. The variable $CD4^+$ T-cell epitope was located and the amino acid substitution responsible for abrogation of $CD4^+$ T-cell recognition was identified (chapter 8). The studies described in this thesis are discussed in the light of evasion of influenza A virus from human T-cell immunity.

Chapter

2

A mutation in the HLA-B*2705-restricted $NP_{383–391}$ epitope affects the human influenza A virus-specific cytotoxic T-lymphocyte response *in vitro*

E.G.M. Berkhoff • A.C.M. Boon • N.J. Nieuwkoop • R.A.M. Fouchier
K. Sintnicolaas • A.D.M.E. Osterhaus • G. F. Rimmelzwaan

Institute of Virology and WHO National Influenza Center, Erasmus Medical Center, Rotterdam, and Laboratory for Histocompatibility and Immunogenetics, Sanquin Bloodbank, Dordrecht, The Netherlands

Journal of Virology 2004; 78:5216-22

Viruses can exploit a variety of strategies to evade immune surveillance by cytotoxic T-lymphocytes (CTL), including the acquisition of mutations in or adjacent to CTL epitopes. Recently, an amino acid substitution (R384G) in an HLA-B*2705-restricted CTL epitope in the influenza A virus nucleoprotein ($NP_{383-391}$; SRYWAIRTR), was associated with escape from CTL-mediated immunity. The effect of this mutation on the *in vitro* influenza A virus-specific CTL response was studied. To this end, two influenza A viruses, with or without the $NP_{383-391}$ epitope, were constructed by reverse genetics and designated influenza virus A/NL/94-384R and A/NL/94-384G respectively. The absence of the HLA-B*2705-restricted CTL epitope in the influenza virus A/NL/94-384G was confirmed in ^{51}Cr-release assays using a T-cell clone specific for the $NP_{383-391}$ epitope. In addition, PBMC stimulated with influenza virus A/NL/94-384G failed to recognize HLA-B*2705 positive target cells pulsed with the original $NP_{383-391}$ peptide. The proportion of virus-specific $CD8^+$ IFN-γ^+ T-cells in *in vitro* stimulated PBMC was determined by intracellular IFN-γ-staining after re-stimulation with virus-infected autologous B-lymphoblastoid cell lines (BLCL) and C1R cell lines expressing only HLA-B*2705. In PBMC stimulated *in vitro* with influenza virus A/NL/94-384G, obtained from several HLA-B*2705 positive donors, the proportion of virus-specific $CD8^+$ T-cells was lower than in PBMC stimulated with influenza virus A/NL/94-384R. This finding indicated that amino acid variation in CTL epitopes can affect the virus-specific CTL response and that the $NP_{383-391}$ epitope is the most important HLA-B*2705-restricted epitope in the NP of influenza A viruses.

Introduction

Cytotoxic T-lymphocytes (CTL) play an important role in the control of viral infections, including those caused by influenza viruses. In mice it has been shown that CTL contribute to protective immunity against influenza viruses of various subtypes (143). Also in humans influenza A virus specific CTL were found to reduce virus titers in the lung and morbidity upon infection (168). To evade host CTL responses, viruses have developed a variety of mechanisms to prevent recognition by specific CTL (138, 183). These mechanisms involve MHC class I-downregulation, as has been described for the human immunodeficiency virus HIV-1 Nef protein (49, 223) and the adenovirus E3/19K protein (34), and blocking of antigen presentation of viral epitopes, as described for certain herpesvirus proteins (126, 134). Alternatively, viruses can accumulate mutations in or adjacent to CTL epitopes affecting peptide processing and presentation, binding to MHC class I-molecules and/or recognition by specific T-cells (102, 280). This escape mechanism has been described predominantly for chronic virus infections, such as those caused by lymphocytic choriomeningitis virus (171, 195), Epstein-Barr virus (EBV) (6, 37, 55, 56, 95), human immunodeficiency virus (HIV) (29, 51, 96, 97, 138, 170, 191, 200, 201), hepatitis B virus (20, 21) and hepatitis C virus (41,

264). Recently, we have identified amino acid sequence variation in CTL epitopes in the nucleoprotein (NP) of influenza A viruses. In the HLA-B*3501-restricted $NP_{418-426}$ epitope, variation was observed in the T-cell receptor contact residues (26). These variants emerged in a chronological order and CTL specific for older variants failed to recognize the mutated version of the same epitope. In addition, amino acid sequence variation was observed at position 384 of the NP. An arginin at this position is an anchor residue for the HLA-B*0801-restricted epitope $NP_{380-388}$ ELRSRYWAI and the HLA-B*2705-restricted epitope $NP_{383-391}$ SRYWAIRTR (123). The observed R384G mutation resulted in the loss of the anchor residues and as a result abolished recognition by CTL (211, 253). Although the rapid fixation of these mutations were explained by small selective advantages and population dynamics in a theoretical model (88), it is unclear to what extent a single amino acid substitution in a CTL epitope affects the overall virus-specific CTL response in humans.

In the present study this issue was addressed *in vitro* using PBMC of HLA-B*2705 positive individuals and genetically engineered recombinant influenza viruses containing an arginin or a glycin at position 384 (but further identical), thus with or without the HLA-B*2705-restricted epitope $NP_{383-391}$ SRYWAIRTR. Using these viruses for the stimulation of PBMC, the *in vitro* CTL response was assessed. It was found that the R384G substitution impaired the influenza virus-specific CTL response *in vitro* significantly.

Material and methods

Plasmids and site directed mutagenesis

For the generation of recombinant influenza viruses, RNA was extracted from culture supernatants containing influenza virus A/Netherlands/18/94 (A/NL/18/94), using a High Pure RNA Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). The RNA was used in a single-tube RT-PCR reaction to amplify viral NP segments. After annealing of the primers AGCAAAAGCAGGGT and AGTAGAAACAAGGGTATTTTTC, first strand synthesis was carried out in 50 µl volumes of 20 mM Tris-HCl buffer pH 8.8, containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml Bovine Serum Albumin (BSA), 10 mM dNTPs, 10 mM DTT, RNAsin, 5 IU Superscript II reverse transcriptase and 5 IU Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). After an incubation for 45 minutes at 42 °C, the mixture was heated at 95 °C for 3 minutes followed by 40 cycles of denaturation (1 min at 95 °C), annealing (2 min at 37 °C) and elongation (3 min at 72 °C). For the addition of Bsa I restriction sites an additional amplification of 30 cycles was performed with primers CTAGGTCTCTTATTAGTAGAAACAAGG and GGGAGGTCTCCGGCCAGCAAAAGCAGG. The amplicon was purified by electroforesis in agarose gels according to standard methods and inserted between the human pol I promotor and the hepatitis delta ribozyme sequence of plasmid pSP72-PhuThep (58).

For site directed mutagenesis, the coding sequence of NP gene of influenza virus A/NL/18/94 was amplified by PCR using primers CAGCGGCCGCATGGCGTCCCAAGGC

and CACTCGAGTTAATTGTCGTACTCCTCTGC and cloned into pBluescript (Stratagene) after digestion with Not I and Xho I. Using this plasmid as a template, site directed mutagenesis was performed by PCR in order to obtain an arginin at position 384 in stead of a glycin (G384R) as previously described (213). The mutated sequence was exchanged using restriction sites Sph I in the NP gene and Xho I in pBluescript, 3' of the insert. Subsequently the Sac I fragment with the mutation at position 384 was exchanged for Sac I fragments in the genomic constructs of NP genes of influenza A virus A/NL/18/94. The nucleotide sequence of all cloned NP genes were determined using standard procedures as previously described (253) in order to confirm identity of the sequences.

The plasmid pHMG-NP, from which the NP of influenza virus A/PR/8/34 was transcribed, was kindly provided by Dr. P. Palese. The bidirectional reverse genetics plasmids pHW181 through pHW188 for the transcription of viral gene segments of influenza virus A/WSN/33 were kindly provided by Dr. R.G. Webster

Generation of viruses

The unidirectional plasmid of the genomic construct of the NP of A/NL/18/94 was transfected into 293T cells with the bidirectional constructs containing the PB1, PB2, PA, HA, NA, M and NS gene segments of A/WSN/33 and pHMG-NP expressing the NP of PR/8/34. For this purpose, 10^6 293T cells were cultured O/N in DMEM (Cambrex, East Rutherford, NJ) supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (antibiotics) in gelatin-coated 10 cm petri dishes. The cells were then transfected with 5 µg of each of the plasmids using the calcium phosphate precipitation method as described previously (58). After 24 hours, the cells were washed with PBS, and 10 ml DMEM containing 2% FCS was added. After another 24 hours the culture supernatant of the transfected 293T cells were harvested and the infectious virus titers were determined as previously described (208). Titers of 10^3 $TCID_{50}$/ml were obtained with A/NL/94-384G and A/NL/94-384R constructs routinely. Subsequently, virus stocks were prepared by infecting confluent Madin-Darby-Canine-Kidney (MDCK) cells in 162 cm^2 flasks with 1 ml supernatant of the transfected 293T cells and 4 ml EMEM medium (Cambrex) supplemented with 4% BSA, 0.02 M Hepes, 0.01 M $NaHCO_3$, antibiotics and 0.1% trypsin (infection medium) at 37 °C for 1 hour. The cells were washed once with PBS and subsequently cultured at 37 °C in infection medium. After three days, culture supernatants were harvested, cleared by low speed centrifugation, aliquoted and stored at –70 °C until use. The viruses with or without the $NP_{383-391}$ epitope were designated A/NL/94-384R and A/NL/94-384G respectively. Upon infection of MDCK cells virus titers were amplified upto 10^7 $TCID_{50}$/ml for both viruses.

Peripheral Blood Mononuclear Cells (PBMC)

PBMC from healthy HLA-B*2705 positive blood donors were isolated from heparinized blood (Sanquin Bloodbank of Rotterdam) by density gradient centrifugation using Lymphocyte Separation Medium (ICN Biomedicals/Cappel, Aurora, OH) and cryopreserved at –135 ºC. Genetic subtyping was performed in the laboratory for Histocompatibility and Immunogenetics at the Sanquin Bloodbank using a commercial typing system (GenoVision, Vienna, Austria).

In vitro stimulation of PBMC with influenza A viruses

PBMC were resuspended in RPMI 1640 medium with 25 mM hepes buffer and L-glutamine (Cambrex) supplemented with 10% FCS and antibiotics (R10F). Five million PBMC were infected with influenza viruses A/NL/94-384G, A/NL/94-384R or Resvir-9, a reassortant virus between strains A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2), containing the NP, HA and NA of A/Nanchang/933/95, at a multiplicity of infection (MOI) of three in a volume of 5 ml R10F as described previously (25). After one hour at 37 ºC, the cells were resuspended in RPMI 1640 medium supplemented with 10% human AB serum (Sanquin Bloodbank), antibiotics and 20 μM β-mercaptoethanol (R10H) and added to non-infected PBMC at a ratio of 1:1 in a 25 cm^2 culture flask. After 48 hours of stimulation rIL-2 was added to a final concentration of 50 IU/ml. After an additional 7-8 days the cells were used as effector cells in the ^{51}Cr-release assays and for the enumeration of virus-specific $CD8^+$ T-cells by the intracellular interferon-gamma (IFN-γ) staining (see below).

$CD8^+$ T-cell clones

The generation of a $CD8^+$ T-cell clone directed against the HLA-B*2705-restricted epitope $NP_{383-391}$ derived from the NP was described previously (253). The $CD8^+$ T-cell clone directed against the immunodominant and conserved HLA-A*0201-restricted epitope $M1_{58-66}$ derived from the matrix protein (M1) was established according to similar procedures.

Isolation of $CD8^+$ T-cells

$CD8^+$ T-cells were isolated from the effector cell populations by magnetic sorting, using a $CD8^+$ cell selection kit (Dynal Biotech GmbH, Hamburg, Germany). The cells were washed once in PBS supplemented with 2% FCS (P2F) and subsequently resuspended in P2F at a concentration of 10^7/ml. Capture dynabeads were added to the cell suspension at a dynabead to $CD8^+$ T-cell ratio of 8:1. After 30 minutes incubation on ice, the dynabeads/cells were washed five times with 5.0 ml P2F. The dynabeads, together with the attached cells, were reconstituted in 200 μl RPMI 1640 medium supplemented with 1% FCS (R1F). To detach the cells from the dynabeads, 20 μl of DETACHaBEAD® (Dynal Biotech GmbH) was added.

After 1 hour incubation at 20 ºC, the released cells were isolated, washed once in R10F and used as effector cells in ^{51}Cr-release assays using C1R cells as target cells.

Target cells

Autologous B-lymphoblastoid cell lines (BLCL), produced as described previously (212), and two C1R cell lines, kindly provided by Dr. P. Romero (C1R cell line) and Dr. J. Lopez de Castro (HLA-B*2705-transfected C1R cell line), were used as target cells. Peptide labeling was performed by incubating the cells overnight with 5 μM peptide per 10^6 cells in 1 ml R10F. Peptides were manufactured, HPLC-purified and analyzed with mass spectrometry by Eurogentec (Seraing, Belgium). For exogenous protein labeling 50 μg recombinant influenza virus nucleoprotein (rNP), derived from influenza virus A/HK/2/68 (rNP-HK) or A/NL/18/94 (rNP-NL), was added to 10^6 cells in 1 ml R10F, as described previously (254). For infection with influenza virus A/NL/94-384G and A/NL/94-384R, the target cells were infected at a MOI of three in a volume of 1 ml. After an incubation for one hour at 37 ºC the cells were resuspended in R10F and incubated for 16-18 hrs. BLCL and C1R cells were equally susceptible to infection with influenza virus A/NL/94-384G and A/NL/94-384R as determined by immuno fluorescence assays (IFA), using a FITC-conjugated monoclonal antibody (MAb; DAKO, Glostrup, Denmark) directed to the NP.

^{51}Cr-release assay

The target cells were resuspended in RPMI 1640 medium supplemented with 0.1% BSA and antibiotics (R0.1B). Next, $5{\cdot}10^5$ target cells were labeled with 50 μCi $Na_2[^{51}Cr]O_4$ in R0.1B for 1 hr at 37 ºC. After incubation, the cells were washed three times with R10F and adjusted to a concentration of 10^5/ml. Subsequently, 50 μl of the target cells were incubated with PBMC stimulated *in vitro* with influenza virus A/NL/94-384G or A/NL/94-384R at effector-to-target (E:T) ratios of 80, 40, 20, 10, 5, or purified $CD8^+$ T-cell populations or T-cell clones at E:T ratios of 10, 5, 2.5, and 1. Target cells were also incubated with 100 μl of 10% Triton X-100 or R10F to determine the maximum and spontaneous release respectively. After 4 hrs of incubation at 37 ºC, the supernatants were harvested (Skatron instruments, Sterling, Va., USA) and radioactivity was measured by gamma-counting. The percentage specific lysis was calculated with the following formula: (experimental release-spontaneous release) / (maximum release – spontaneous release) × 100%. The ^{51}Cr-release assays were performed in triplicate or quadruplicate and the data are presented as the average.

Intracellular IFN-γ staining and flow cytometry

PBMC expanded *in vitro* after stimulation with influenza viruses A/NL/94-384G or A/NL/94-384R, were resuspended and adjusted to a concentration of 10^6 cells/ml in R10F supplemented with Golgistop (monensin; Pharmingen, Alphen a/d Rijn, The Netherlands).

Hundred thousand effector cells were incubated with $2 \cdot 10^5$ stimulator cells, which were infected, incubated with rNP, peptides or left untreated, for 6 hours at 37 ºC in U-bottom plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands). Subsequently intracellular IFN-γ-staining was performed as described previously (28). In brief, the cells were washed with PBS containing 2% FCS (P2F) and Golgistop, stained with MAb directed to CD3 (Becton Dickinson, Alphen a/d Rijn, Netherlands) and CD8 (Dako), fixed and permeabilized with cytofix and cytoperm (Pharmingen) and stained with a MAb specific for IFN-γ (Pharmingen). At least 10^3 gated $CD3^+$ $CD8^+$ events were acquired using a FACSCalibur (Becton Dickinson) flowcytometer. The data were analyzed using the software program Cell Quest Pro (Becton Dickinson). The data were expressed as the percentage of virus-specific $CD8^+$ IFN-γ^+ T-cells in PBMC cultures and as the percentage of virus-specific IFN-γ^+ cells in the $CD8^+$ T-cell fraction. The relative reduction of virus-specific $CD8^+$ IFN-γ^+ response was calculated according to the formula: (100 – ((% $CD8^+$ IFN-γ^+ T-cells after re-stimulation with influenza virus A/NL/94-384G × 100) / % $CD8^+$ IFN-γ^+ T-cells after re-stimulation with influenza virus A/NL/94-384R)). Routine staining with MAb was carried out with $1\text{-}5 \cdot 10^5$ cells for 30 minutes at 4 ºC. This way the proportion of $CD3^+$, $CD4^+$ and $CD8^+$ cells was determined.

Statistical analysis

It was assumed that the obtained data was not normally distributed, because of the use of different blood donors. Therefore, statistical analysis was performed on the difference in response to influenza virus A/NL/94-384G and A/NL/94-384R infected target cells between A/NL/94-384G and A/NL/94-384R stimulated PBMC by using oneway ANOVA analysis.

Results

Validation of infection with influenza viruses A/NL/94-384G and A/NL/94-384R *in vitro*

To verify that target cells were equally susceptible to infection with influenza virus A/NL/94-384G and A/NL/94-384R, an IFA was performed using BLCL infected with either virus. It was found that the number of infected cells was similar as determined by positive staining for NP (data not shown). In addition, the expression of MHC class I-bound epitopes on the surface of BLCL infected with influenza A virus A/NL/94-384G and A/NL/94-384R and the subsequent recognition by CTL was compared to confirm that it was in the same order of magnitude (figure 1). To this end, ^{51}Cr-release assays were performed using HLA-A*0201, -B*2705 positive BLCL as target cells and $CD8^+$ T-cell clones specific for the conserved epitope from the matrix protein ($M1_{58\text{-}66}$, restricted by HLA-A*0201) and the HLA-B*2705-restricted $NP_{383\text{-}391}$ epitope. As expected, the CTL clone specific for the original $NP_{383\text{-}391}$ epitope recognized target cells infected with influenza virus A/NL/94-384R,

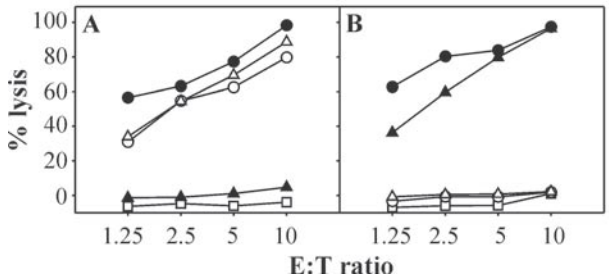


Figure 1. Recognition of BLCL infected with recombinant influenza viruses by CTL clones. HLA-A*0201, -B*2705 positive BLCL were infected with influenza virus A/NL/94-384G (○) or A/NL/94-384R (●), pulsed with $M1_{58-66}$ (△) or $NP_{383-391}$ (▲) peptide or left untreated (□), and used as target cells for $CD8^+$ T cell clones specific for the HLA-A*0201-restricted $M1_{58-66}$ epitope (A) and HLA-B*2705-restricted $NP_{383-391}$ epitope (B) in a ^{51}Cr-release assay. CTL clones were added at different effector-to-target cell ratios as indicated, and specific lysis was calculated.

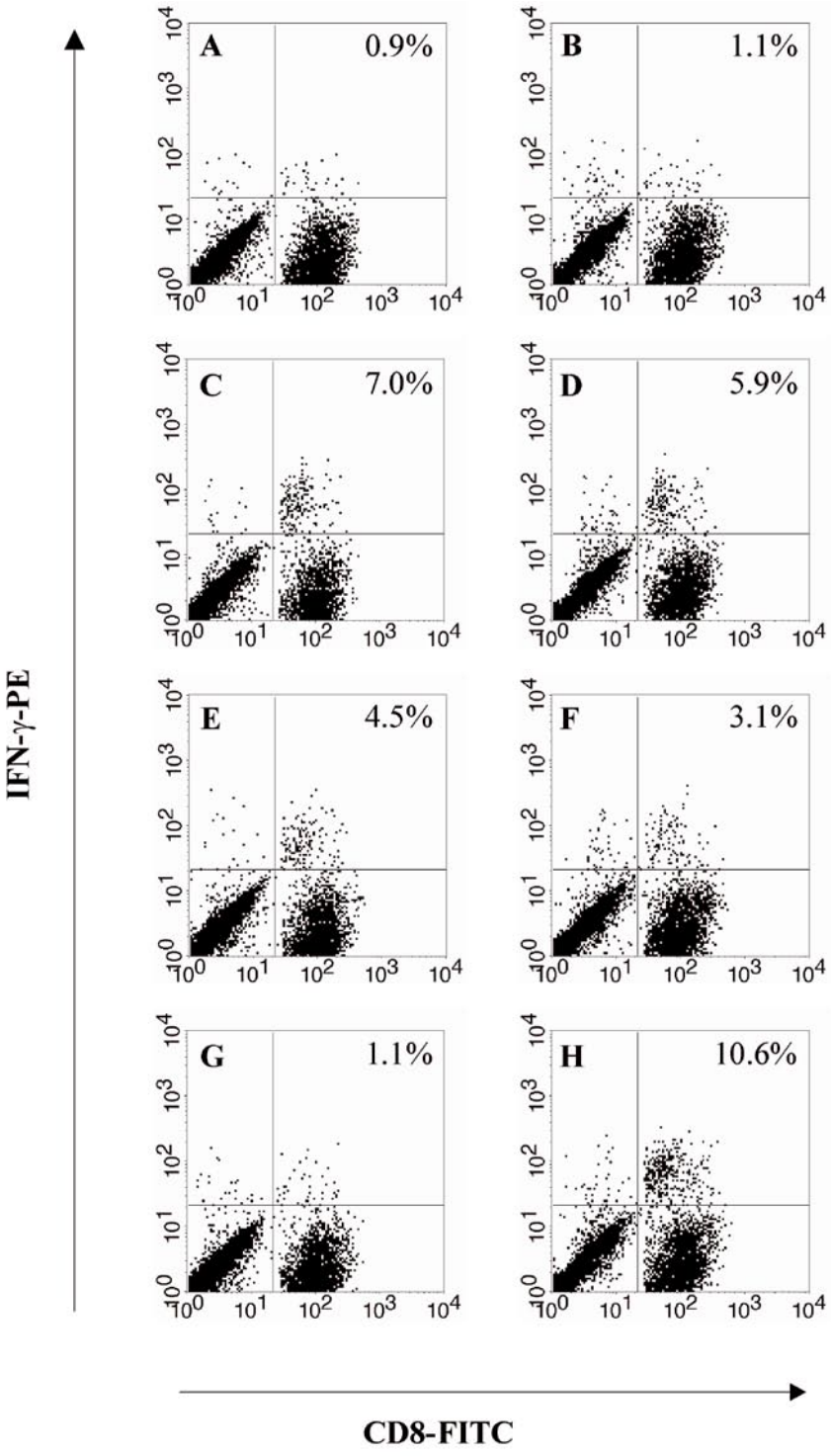
and failed to recognize those infected with influenza virus A/NL/94-384G (figure 1B). In contrast, the CTL clone specific for $M1_{58-66}$ recognized A/NL/94-384G and A/NL/94-384R infected target cells equally well, indicating that the infection of cells, the processing and presentation of immunogenic peptides was comparable for both viruses (figure 1A).

I*n vitro* stimulation of PBMC with recombinant influenza viruses

The viruses A/NL/94-384G and A/NL/94-384R were used for the stimulation of PBMC obtained from an HLA-A*0201, -B*2705 positive blood donor to demonstrate that the R384G mutation resulted in the depletion of a CTL response against the $NP_{383-391}$ epitope. As shown in figures 2A-H, stimulation with A/NL/94-384G (A,C,E and G) or A/NL/94-384R (B,D,F and H) resulted in similar numbers of IFN-γ^+ $CD8^+$ T-cells specific for the conserved HLA-A*0201-restricted $M1_{58-66}$ epitope and the HLA-B*2705-restricted $NP_{174-184}$ epitope measured after re-stimulation with peptide pulsed BLCL. In contrast, the response to $NP_{383-391}$ was virtually absent in PBMC cultures stimulated with influenza virus A/NL/94-384G, but not in those stimulated with influenza virus A/NL/94-384R. In the PBMC of donor 2, 10.6% of the $CD8^+$ T-cells were specific for the $NP_{383-391}$ epitope (figure 2H). Similar results were observed for the other donors. The absence of $CD8^+$ T-cells specific for $NP_{383-391}$ epitope as measured by intracellular IFN-γ staining coincided with the lack of capacity of the PBMC culture to lyse target cells pulsed with the $NP_{383-391}$ peptide, but not of those pulsed with the $M1_{58-66}$ peptide or the $NP_{174-184}$ peptide (figure 2I and J).

Magnitude of the influenza virus-specific CTL response *in vitro*

The contribution of the $NP_{383-391}$ epitope to the influenza A virus-specific CTL response was determined by measuring the proportion of $CD3^+$ $CD8^+$ IFN-γ^+ cells in PBMC cultures stimulated with influenza viruses A/NL/94-384G or A/NL/94-384R. IFN-γ expression was induced by re-stimulation with autologous BLCL or C1R-B27 cells infected with the respec-


A
0.9%
B
1.1%
C
7.0%
D
5.9%
E
4.5%
F
3.1%
G
1.1%
H
10.6%
IFN-γ-PE
CD8-FITC

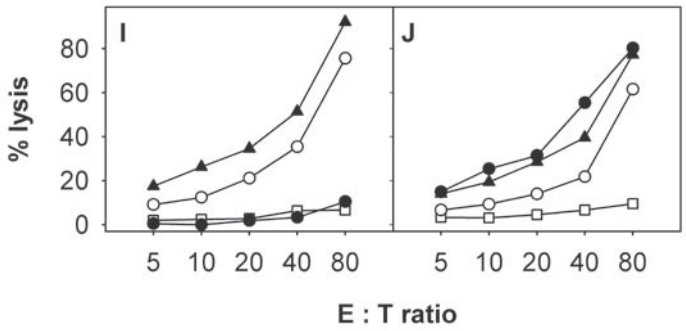


Figure 2. IFN-γ expression and lysis by $CD3^+$ $CD8^+$ cells after stimulation of PBMC with influenza virus A/NL/94-384G or A/NL/94-384R. PBMC of an HLA-A*0201, -B*2705 positive donor, expanded after stimulation with influenza virus A/NL/94-384G (A,C,E and G) and A/NL/94-384R (B,D,F and H), were re-stimulated with BLCL pulsed with $M1_{58-66}$ epitope (C and D), $NP_{174-184}$ epitope (E and F), or $NP_{383-391}$ epitope (G and H). Re-stimulation with untreated cells was used as negative controls (A and B). Virus-specific CTL were visualized after staining with MAb specific for CD3, CD8 and IFN-γ (A-H). Indicated is the percentage IFN-γ^+ cells within the $CD8^+$ T cell population. CTL specific for $M1_{58-66}$ epitope (▲), $NP_{174-184}$ epitope (○), and $NP_{383-391}$ epitope (●) were also detected by ^{51}Cr-release assay, using peptide pulsed BLCL as target cells and PBMC cultures stimulated with A/NL/94-384G (I) or A/NL/94-384R (J). Untreated cells were included as negative controls (□). Effector cells were added at different effector-to-target cell ratios as indicated, and specific lysis was calculated.

tive influenza viruses or incubated with rNP or peptides. Figure 3 shows such an analysis for donor 2 after re-stimulation with autologous BLCL. A response was observed after re-stimulation with BLCL incubated with the $NP_{383-391}$ peptide or rNP-HK in PBMC cultures expanded after stimulation with influenza virus A/NL/94-384R. Such a response was not observed after re-stimulation with rNP-NL, which lacks the $NP_{383-391}$ epitope (figure 3B). After primary stimulation with influenza virus A/NL/94-384G, no response was observed after re-stimulation with the $NP_{383-391}$ peptide or rNP (figure 3A). In addition, no differences were observed with the influenza virus A/NL/94-384G stimulated culture in the *in vitro* recall response induced by influenza viruses A/NL/94-384G and A/NL/94-384R. However, the CTL response against influenza virus A/NL/94-384G was significantly lower than the response to A/NL/94-384R in PBMC cultures that were stimulated with influenza virus A/NL/94-384R. These differences are depicted for all donors tested as the percentage of virus-specific $CD8^+$ IFN-γ^+ T-cells in the PBMC cultures (figure 4A) and as the percentage of virus-specific IFN-γ^+ cells in the $CD8^+$ T-cell fraction (figure 4B), ranging from 1-9% and 1-20% respectively. Since no differences were observed after primary stimulation with a virus lacking the $NP_{383-391}$ epitope, these results show that depletion of this epitope resulted in a substantial decrease of the influenza A virus-specific CTL response *in vitro*. The relative reduction in CTL response induced by the virus without the $NP_{383-391}$ epitope compared to the response against the virus containing the $NP_{383-391}$ epitope ranged from 2-46% with a median of 30% (figure 4C).

When C1R-B27 cells were used for the re-stimulation of PBMC cultures, stimulated with influenza virus A/NL/94-384G or A/NL/94-384R, comparable results were obtained (data not shown). As expected, the percentage of virus-specific CTL restricted by a single

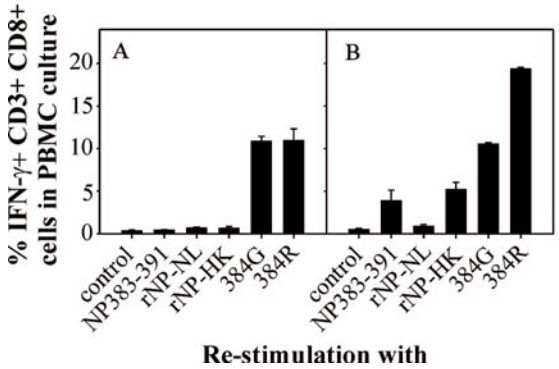


Figure 3. Percentage of virus-specific $CD8^+$ T cells in PBMC cultures stimulated *in vitro*. The percentage of $CD8^+$ IFN-γ^+ T cells was determined in PBMC of donor 2 after stimulation *in vitro* with influenza virus A/NL/94-384G (A) and A/NL/94-384R (B), without and with the HLA-B*2705-restricted $NP_{383-391}$ epitope respectively. Expanded cells were re-stimulated with autologous BLCL cells that were infected with influenza viruses A/NL/94-384G or A/NL/94-384R, or that were incubated with peptide ($NP_{383-391}$; SRYWAIRTR) or rNP derived from influenza virus A/HK/2/68 (rNP-HK) or A/NL/18/94 (rNP-NL) as indicated. Virus-specific CTL were visualized after staining with MAb specific for CD3, CD8 and IFN-γ. The data represent the proportion of $CD3^+$ $CD8^+$ IFN-γ^+ cells of the total PBMC cultures. These values were calculated as follows: (% IFN-γ^+ cells in $CD3^+$ $CD8^+$ fraction) × (% $CD8^+$ T cells in PBMC culture).

HLA allele was lower than the percentage measured after re-stimulation with autologous BLCL. On average the HLA-B*2705-restricted response constituted 65% of the response measured over all HLA alleles using the autologous BLCL.

The PBMC cultures stimulated with influenza viruses A/NL/94-384G and A/NL/94-384R were also tested in parallel in ^{51}Cr-release assays. In contrast to the results obtained with intracellular IFN-γ staining, no clear differences were observed between lysis of target cells infected with influenza virus A/NL/94-384G or A/NL/94-384R by PBMC expanded after stimulation with influenza virus A/NL/94-384R (figure 5A,B). However, when protein labeled BLCL or C1R-B27 cells were used as target cells, it was found that PBMC cultures stimulated with influenza virus A/NL/94-384R recognized target cells labeled with rNP-HK, but failed to recognize target cells labeled with rNP-NL, containing the R384G mutation (figure 5D). PBMC cultures stimulated with influenza virus A/NL/94-384G failed to recognize protein labeled target cells at all (figure 5C). These findings indicate that the $NP_{383-391}$ epitope constituted an important CTL epitope recognized in the NP-specific CTL response by these donors.

Discussion

In the present study the effect of a single amino acid substitution in epitope $NP_{383-391}$ on the *in vitro* human CTL response specific for influenza A virus was investigated, using recombinant influenza viruses differing only at position 384 of the NP. Using these influenza viruses

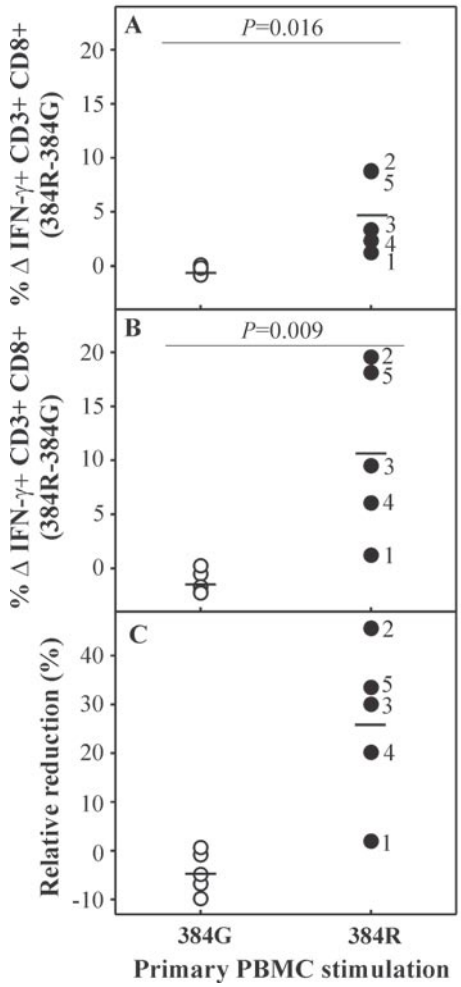


Figure 4. Reduction in *in vitro* CTL response by loss of the $NP_{383-391}$ epitope. The reduction (Δ) in the % virus-specific $CD8^+$ IFN-γ^+ T cells in PBMC cultures stimulated with influenza viruses A/NL/94-384G or A/NL/94-384R is shown for all 5 donors (A). The reduction was calculated as % $CD8^+$ IFN-γ^+ T cells after re-stimulation with influenza virus A/NL/94-384R minus % $CD8^+$ IFN-γ^+ T cells after re-stimulation with influenza virus A/NL/94-384G. The % $CD8^+$ IFN-γ^+ T cells in PBMC cultures were determined as indicated in figure 3. The reduction was also expressed as the difference in % virus-specific IFN-γ^+ T cells within the $CD3^+$ $CD8^+$ fraction of the PBMC cultures, as measured by flow cytometry (B). In panel C, the reduction of the % virus-specific $CD8^+$ IFN-γ^+ T cells in PBMC cultures was calculated as the relative reduction: (100 – ((% $CD8^+$ IFN-γ^+ T cells after re-stimulation with influenza virus A/NL/94-384G × 100) / % $CD8^+$ IFN-γ^+ T cells after re-stimulation with influenza virus A/NL/94-384R)). In all three panels the average is shown (—). Numbers refer to the donors listed in table 2.

for the stimulation of PBMC obtained from HLA-B*2705 positive individuals, it was found that the mutation reduced the magnitude of the virus-specific CTL response *in vitro*.

Before influenza viruses A/NL/94-384G and A/NL/94-384R were compared for their capacity to induce CTL responses *in vitro*, it was confirmed that BLCL and C1R cells were equally susceptible to infection with these two viruses and that antigen processing

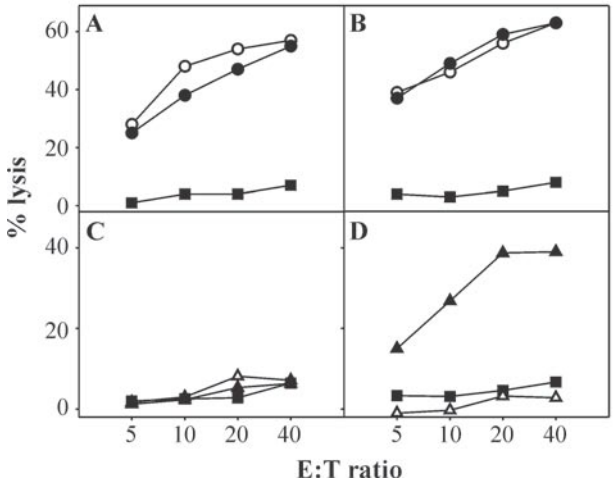


Figure 5. Recognition of BLCL infected with influenza viruses (A and B) or incubated with rNP (C and D) by *in vitro* stimulated PBMC obtained from donor 2. PBMC expanded after stimulation with influenza virus A/NL/94-384G (A,C) or A/NL/94-384R (B,D) were used as effector cells in ^{51}Cr-release assays. Autologous BLCL infected with influenza virus A/NL/94-384G (○) or A/NL/94-384R (●) or BLCL incubated with rNP derived from influenza virus A/NL/18/94 (△) or A/HK/2/68 (▲) were used as target cells. Untreated cells were included as a negative control (■). The data are presented as the percentage specific lysis at the indicated E:T ratios.

and presentation of MHC class I-peptide complexes on the surface of these cells were in the same order of magnitude, using a CTL clone specific for the $M1_{58-66}$ epitope. Furthermore, the presence and absence of the $NP_{383-391}$ epitope in influenza viruses A/NL/94-384R and A/NL/94-G respectively was confirmed, using a $NP_{383-391}$ specific CTL clone and PBMC of HLA-B*2705 positive individuals stimulated *in vitro* with these viruses.

Stimulation of HLA-B*2705 positive PBMC with influenza virus A/NL/94-384G did not result in stronger responses against the conserved epitopes $NP_{174-184}$ and $M1_{58-66}$, than after stimulation with influenza virus A/NL/94-384R, indicating that the loss of one HLA-B*2705-restricted epitope was not compensated by the response to another in this *in vitro* system. In PBMC cultures of five HLA-B*2705 positive individuals, expanded after stimulation with influenza virus A/NL/94-384R, $CD8^+$ T-cells specific for $NP_{383-391}$ peptide and rNP-HK, but not rNP-NL, were demonstrated. The results obtained with rNP-HK or rNP-NL in intracellular IFN-γ staining and ^{51}Cr-release assays, indicated that the $NP_{383-391}$ is the most immunodominant epitope in the NP restricted by HLA-B*2705 and other alleles expressed in the individuals tested (HLA-A*0101, 0201, 0301, 1101, 2301, 3101 and HLA-B*0801, 4101; tables 1 and 2). The response to influenza virus A/NL/94-384G was similar in influenza virus A/NL/94-384G and A/NL/94-384R stimulated PBMC. However, in influenza virus A/NL/94-384R stimulated PBMC a lower frequency of influenza virus A/NL/94-384G specific cells was observed than influenza virus A/NL/94-384R specific cells. This difference was used as a measure for the reduction in virus-specific CTL response by the loss of the $NP_{383-391}$ epitope. In four out of five donors tested, a significant reduction in

Table 1. Known influenza A virus CTL epitopes likely recognized by the five individuals used in the present study.

HLA Restriction	Protein (amino acids)	Amino acid sequence
A*01	PB1 (591-599)	VSDGGPNLY
	NP (44-52)	CTELKLSDY
A*0201	M1 (58-66)	GILGFVFTL
	NS1 (122-130)	AIMDKNIIL
	NA (213-221)	CVNGSCFTV
	PA (46-54)	FMYSDFHFI
	PA (225-233)	SLENFRAYV
	PB1 (413-421)	NMLSTVLGV
	NA (75-84)	SLCPIRGWAI
A*03	NP (265-273)	ILRGSVAHK
	M1 (27-35)	RLEDVFAGK
A*1101	HA (63-70)	GIAPLQLGK
	HA (149-158)	VTAACSHAGK
	HA (450-460)	RTLDFHDSNVK
	M1 (13-21)	SIIPSGPLK
	NP (188-198)	TMVMELVRMIK
B*08	NP (380-388)	ELRSRYWAI
B*2705	NP(383-391)	SRYWAIRTR
	NP(174-184)	RRSGAAGAAVK

Adapted from the Influenza Sequence Database (www.flu.lanl.gov) (158).

the number of influenza A virus-specific $CD8^+$ CTL was measured. Thus, the loss of a single epitope can have a major impact on the CTL response *in vitro*. The absence of a reduction in donor 1 correlated with the subdominant nature of the $NP_{383-391}$ epitope in this study subject. Overall, there was a correlation between the frequency of $NP_{383-391}$ specific CTL in the PBMC cultures stimulated with A/NL/94-384R, and the reduction in CTL response after stimulation with an influenza virus lacking the $NP_{383-391}$ epitope.

Since three of the five subjects tested were also HLA-B*0801 positive, it is possible that also the loss of the HLA-B*0801-restricted $NP_{380-388}$ epitope contributed to the reduction of the CTL response in these donors. It should be realized however that the $NP_{380-388}$ epitope is a minor epitope, especially in the presence of an HLA-B*2705-restricted response (25) and therefore its loss could only marginally be responsible for the observed reduction in CTL response.

The differences in CTL responses measured by intracellular IFN-γ staining were not detected in ^{51}Cr-release assays using virus-infected BLCL as target cells. The resolution of the ^{51}Cr-release assay may not be sufficient to detect these differences, considering the reduction in frequency (1-9%) of virus-specific cells in the *in vitro* expanded PBMC, which were used as effector cells in these assays, caused by the deletion of the $NP_{383-391}$ epitope.

Table 2. HLA -A and -B genotype of the five individuals used in the present study and the proportion of virus-specific $CD8^+$ CTL and the proportion of CTL specific for $NP_{383-391}$ in PBMC stimulated with influenza virus A/NL/94-384R

Donor	HLA-A and -B genotype	% of CD8+ T cells specific for:		Relative proportion of
		A/NL/94-384R	$NP_{383-391}$	$NP_{383-391}$
1	A*0101 A*0201 B*0801 B*2705	62.0[1]	4.6[2]	7.4[3]
2	A*0101 A*0201 B*0801 B*2705	42.9	10.0	23.3
3	A*0301 A*2301 B*2705 B*4101	31.6	8.6	27.2
4	A*1101 A*3101 B*2705 B*2705	29.9	5.0	16.7
5	A*0101 A*0201 B*0801 B*2705	42.5	8.6	20.2

Determined by intracellular IFN-γ staining after *in vitro* re-stimulation with influenza virus A/NL/94-384R[1] and after re-stimulation with $NP_{383-391}$ peptide pulsed autologous BLCL[2]. The relative proportion of $NP_{383-391}$ specific $CD8^+$ T cell was calculated according to the formula: (% $NP_{383-391}$ specific / % virus-specific)x100[3]. The average of two independently repeated experiments is given.

Based on these data we conclude that the mutation in the $NP_{383-391}$ epitope impaired the overall *in vitro* CTL response directed against the influenza virus significantly. However, it is not clear from these studies what the impact is of the R384G mutation on the *in vivo* CTL response in humans. Of interest, recently it was demonstrated in a mouse model for influenza virus that deletion of a dominant H-$2D^b$-restricted epitope from the NP ($NP_{366-374}$) of influenza A viruses by site directed mutagenesis, resulted in the loss of a CTL response specific for this epitope *in vivo*. This correlated with a prolonged duration of viral shedding in infected mice and increased mortality rates (259). Furthermore, the loss of an immunodominant epitope was responsible for the prolonged viral shedding (upto two months) in RAG-1 deficient mice transgenic for the T-cell receptor specific for that epitope (202). If infection of humans also results in reduced control of the infection with CTL epitope mutant influenza viruses, it might be expected that HLA-B*2705 positive individuals may suffer more from infection with a mutant virus (lacking the $NP_{383-391}$ epitope) than from a wild type virus. Of interest, prolonged viral shedding in a small proportion of individuals in the human population (e.g. 8% HLA-B*2705 positives) was sufficient to explain the rapid fixation of the R384G substitution in the CTL epitope $NP_{383-391}$ at the population level, using a recently developed theoretical model (88, 253).

Collectively, the data obtained in the present study showed that the loss of an immunodominant epitope by a mutation at an anchor residue affects the CTL response *in vitro* significantly. It could be speculated that the emergence of influenza A viruses with mutations in CTL epitopes have a profound advantage in individuals expressing the corresponding HLA-molecules, eventually leading to the rapid fixation of these mutants (88).

Acknowledgements

This work was supported by Novaflu - EU grant QLRT-2001-01034. We thank W. Levering for performing the HLA-typing and S. Sakko for organizing the blood collection of blood donors (Sanquin Bloodbank). We also acknowledge M.M. Geelhoed-Mieras for excellent technical assistance. Dr. R.A.M. Fouchier is a fellow of the Royal Dutch Academy of Arts and Sciences.

Chapter

3

Functional compensation of a detrimental amino acid substitution in a cytotoxic T-lymphocyte epitope of influenza A viruses by co-mutations

G.F. Rimmelzwaan • E.G.M. Berkhoff • N.J. Nieuwkoop
R.A.M. Fouchier • A.D.M.E. Osterhaus

Department of Virology and WHO National Influenza Center,
Erasmus Medical Center, Rotterdam, The Netherlands

Journal of Virology 2004; 78:8946-9

Influenza A viruses accumulate amino acid substitutions in cytotoxic T-lymphocyte (CTL) epitopes allowing these viruses to escape from CTL immunity. The arginin to glycin substitution at position 384 of the viral nucleoprotein is associated with escape from CTL. Introduction of the R384G substitution in the nucleoprotein gene segment of influenza virus A/Hong Kong/2/68 by site-directed mutagenesis was detrimental to viral fitness. Introduction of one of the co-mutations associated with R384G, E375G, restored viral fitness and NP-functionality partially. We hypothesized that influenza A viruses need to overcome functional constraints to accumulate mutations in CTL epitopes and escape from CTL.

Cytotoxic T-lymphocytes (CTL) play an important role in the control of viral infections, including those caused by influenza viruses (168, 277). Viruses exploit many strategies to evade recognition by virus-specific CTL, including the accumulation of amino acid substitutions in or adjacent to CTL epitopes (138, 183). As a result, the epitope may no longer bind to its corresponding MHC class I-molecule, be liberated from its native protein and/or recognized by CTL specific for that epitope. This escape mechanism has been described predominantly for persistent virus infections. However, also epitopes from the influenza virus nucleoprotein (NP) exhibit amino acid variation associated with escape from recognition by CTL (26, 211, 253). The rapid fixation of these mutations was explained by small selective advantages and population dynamics in a theoretical model, using the R384G mutation in the HLA-B*2705-restricted $NP_{383-391}$ epitope as example (88). The R384G mutation resulted in the loss of the anchor residue and as a result ablated recognition by CTL (211, 253). The loss of this epitope affected the human *in vitro* CTL response significantly (16). During this study, it appeared that the R384G substitution in the NP gene of influenza virus A/Hong Kong/2/68 (A/HK/68) prevented rescue of virus by reverse genetics, indicating that it was detrimental to viral fitness. Consultation of the influenza sequence database at http://www.flu.lanl.gov (158), revealed a number of co-mutations associated with the R384G substitution. We hypothesized that one or more of these co-mutations were functionally compensating the detrimental effect of the R384G mutation. This hypothesis was tested using recombinant influenza viruses with the R384G mutation alone or in combination with one of the co-mutations.

Seven amino acid substitutions were associated with the R384G substitution (table 1). However, some of these co-mutations (E18D, I197V, M239V and S259L) were not exclusively associated with the presence of the R384G substitution, indicating that these additional amino acid substitutions may not be essential for viral fitness. The remaining three substitutions, R65K, D127E and E375G, were tightly associated with the R384G substitution. Of these three, the substitution at position 375 was in closest proximity of the CTL epitope and the effect of this co-mutation was studied on NP functionality and fitness of viruses with and without the R384G mutation. Also in HIV-1 CTL escape mutants, clus-

Table 1. Amino acid substitutions associated with the R384G substitution[a]

Substitution	No. of virus strains with substitution/total (%)	
	384R	384G
18E	46/46 (100%)	2/6 (33%)
65R	46/46 (100%)	0/6 (0%)
127D	46/46 (100%)	0/6 (0%)
197I	46/46 (100%)	1/6 (17%)
239M	46/46 (100%)	2/6 (33%)
259S	130/137 (94%)	0/76 (0%)
375E	136/137 (99.3%)	0/76 (0%)
18D	0/46 (0%)	4/6 (67%)
65K	0/46 (0%)	6/6 (100%)
127E	0/46 (0%)	6/6 (100%)
197V	0/46 (0%)	5/6 (83%)
239V	0/46 (0%)	4/6 (67%)
259L	8[b]/137 (6%)	76/76 (100%)
375G	1/137 (0.7%)	76/76 (100%)

[a] Full-length NP amino acid sequences of influenza virus A/NL/18/94 and 51 influenza A (H3N2) viruses obtained from the influenza sequence database at LANL (158) and a 151 amino acid fragment (aa 240-391) of 162 Dutch influenza A (H3N2) strains (253) were analyzed. Seven amino acid substitutions were identified associated with the R384G substitution.
[b] These 8 viruses with 259L circulated between 1968 and 1973.

tered mutations have been observed with a mutation in an HLA-B27-restricted CTL epitope in the gag protein (133).

For the generation of recombinant influenza viruses with R384 and G384 respectively, the NP gene-segments of influenza viruses A/HK/68 and A/Netherlands/18/94 (A/NL/94) were amplified by RT-PCR as described previously (16) and inserted between the human pol I promotor and the hepatitis delta ribozyme sequence of plasmid pSP72-PhuThep (58). Site-directed mutagenesis of the respective NP genes was performed by PCR as described (213). Sac I fragments (encoding aa 152-465) of the respective NP were exchanged with Sac I fragments in the genomic NP-construct of influenza A virus A/Puerto Rico/8/34 (A/PR/34) to obtain chimeric constructs with A/PR/34-sequences at the distal 5' and 3' ends of the NP gene-segments. The plasmids pHMG-NP, pHMG-PB1, pHMG-PB2, and pHMG-PA encoding the NP and the polymerase proteins PB1, PB2 and PA of influenza virus A/PR/8/34, were kindly provided by Dr. P. Palese (196). The bi-directional reverse genetics plasmids pHW181 through pHW188 for the transcription of viral gene segments of influenza virus A/WSN/33 were kindly provided by Dr. R.G. Webster (114). For the generation of viruses, the NP constructs of A/NL/94 or A/HK/68 were transfected into 293T cells together with pHMG-NP and all A/WSN/33 genomic constructs except the one encoding the NP gene-segment as described (16). Twenty-four hours after transfection virus was passaged in Madin-Darby-Canine-Kidney (MDCK) cells and infectious virus titers were determined as described (208). The viruses were named after the NP construct that was used for their generation (figure 1). Only infectious virus was detected in the culture supernatant

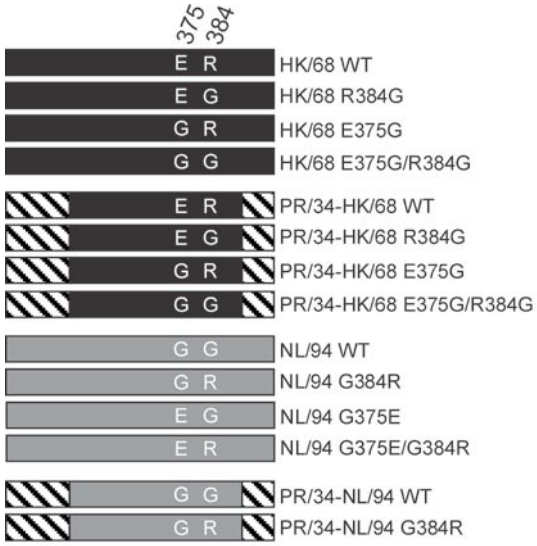


Figure 1. NP constructs used in the present study. For the generation of genomic constructs the NP gene segments of influenza viruses A/HK/68 (black) and A/NL/94 (light gray) were amplified by PCR and cloned under control of the human pol I promotor. Amino acid substition at positions 375 and 384 were introduced by site-directed mutagenesis as indicated. In addition, chimeric NP gene segments were constructed by placing the Sac I fragment encompassing the region encoding amino acids 152-465 of the NP derived from A/HK/68 and A/NL/94 in a background of A/PR/34 (Hatched). WT indicates wild type, un-mutated NP sequences of the respective NP genes.

of 293T cells transfected with plasmids encoding the NP gene-segments of NL/94 WT and NL/94 G384R (data not shown). However, upon passage in MDCK cells, virus replication was observed with the NP gene-segments of HK/68 WT, chimeric PR/34-HK/68 WT, NL/94 and PR/34-NL/94, the latter two with or without the G384R substitution. No virus was detected using constructs containing NP sequences of HK/68 with the R384G substitution (HK/68 R384G or PR/34-HK68 R384G) (Figure 2), confirming that this mutation is detrimental to viral fitness. For A/HK/68, the virus titers obtained after passage in MDCK cells were reproducibly higher with the chimeric constructs than with the full-length A/HK/68 gene-segments, which may be related to more efficient packaging. For A/NL/94 it was the other way around. The reason for this observation is not clear. Introduction of the E375G mutation in the HK/68-R384G or the PR/34-HK/68 NP-R384G gene restored the possibility to rescue virus by reverse-genetics (Figure 2C-D). The titers that were obtained with the E375G/R384G double mutants were not as high as in the original virus, indicating that multiple co-mutations may be required to fully restore viral fitness. The E375G mutation by itself did not influence the fitness of influenza virus A/HK/68 to a great extent. This was also confirmed by assessing the multi-step growth kinetics of these viruses after infection of MDCK cells using equivalent moi's of 0.001 and 0.01 $TCID_{50}$ per cell (figure 2 G and H). The addition of the G375E substitution to the G384R substitution in the NP of A/NL/94 did not affect the fitness of these viruses to a great extent (figure 2B). However, the introduc-

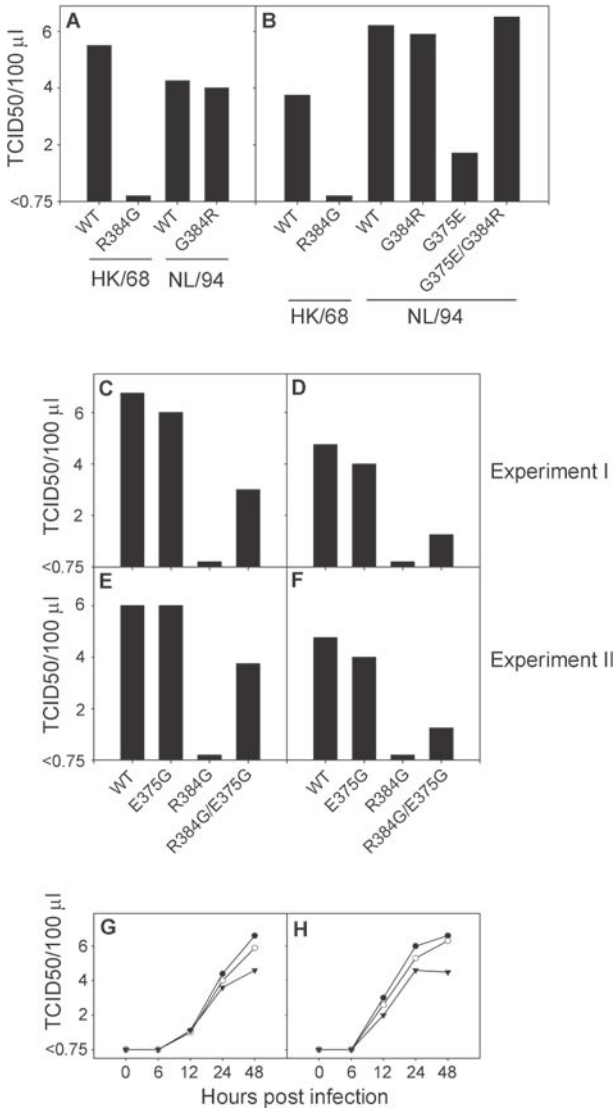


Figure 2. Amino acid substitution R384G is detrimental to fitness of viruses containing the NP sequences of A/HK/68. Upon transfection of 293T cells and subsequent rescue in MDCK cells infectious virus titers were determined of viruses made with chimeric NP gene segments (A) containing sequences obtained from A/PR/34 and A/HK/68 or A/NL/94, with or without reciprocal amino acid substitutions at position 384 as indicated, or full length NP gene segments of A/HK/68 or A/NL/94 (B). The results of a representative experiment are shown. Amino acid substitution E375G restores fitness of viruses with A/HK/68-R384G NP sequences (C-F). Upon transfection of 293T cells and subsequent rescue in MDCK cells infectious virus titers were determined of viruses made with chimeric NP gene segments (C and E) containing sequences obtained from A/PR/34 and A/HK/68 and full length A/HK/68 NP sequences (D and F) without (WT) or with amino acid substitutions E375G and R384G in NP as indicated. The results of two independent experiments are shown. For influenza viruses A/HK/68 WT (●), A/HK/68-E375G (○) and A/HK/68-E375G/R384G (▼) grow curves were determined (G and H) after infection of MDCK cells at a moi of 0.001 (G) or 0.01 (H). The data represent the average of two experiments.

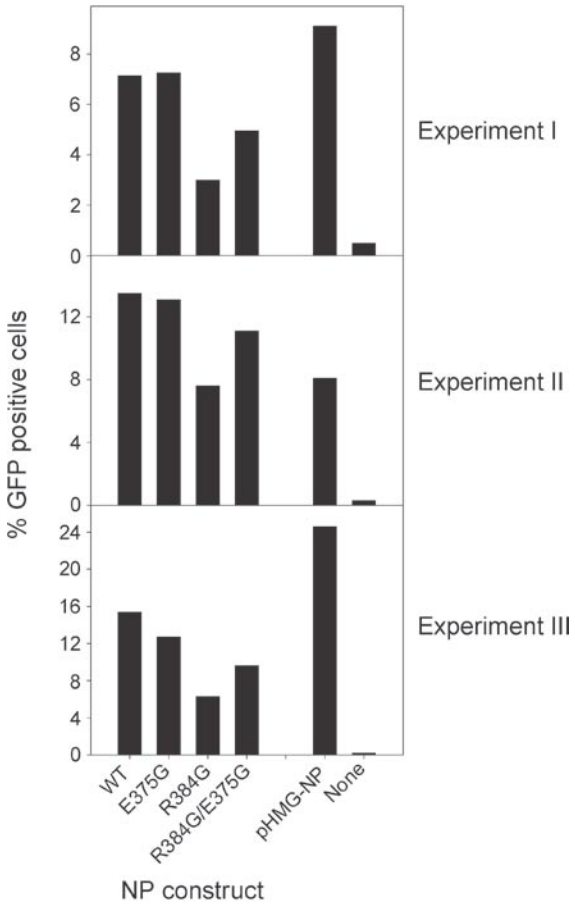


Figure 3. Functional analysis of A/HK/68 NP with or without amino acid substitution at positions 375 and 384. Functionality of the NP was tested in a trans complementation assay. 293T cells were transfected with plasmids pHMG-PB1, pHMG-PB2, pHMG-PA, RF419 and a plasmid encoding the NP of A/HK/68 with or without substitutions at positions 375 and 384 as indicated. Cells transfected with plasmid pcDNA3 served as negative control, while cells co-transfected with pHMG-NP was used as positive control. The results are shown as the percentage of cells showing GFP-expression as measured by flow cytometry. The results of three independent experiments are shown. With the plasmid pEGFP-N1 typically transfection-efficiencies of >95% were obtained.

tion of the G375E substitution alone was detrimental to viral fitness. This confirms that the 375E/384G combination affects viral fitness as was already observed with the A/HK/68 NP constructs. A NP-transcomplementation assay was performed as described previously (253) to correlate differences in viral fitness with functionality of the NP variants. The NP encoding sequences of influenza virus A/HK/68 variants were cloned into a modified version of the eukaryotic expression plasmid pcDNA3.

These plasmids were transfected into 293T cells with plasmids pHMG-PB1, pHMG-PB2 and pHMG-PA and RF419, from which the green fluorescent protein (GFP) gene flanked with the influenza A virus noncoding region of the NS gene-segment is transcribed

in a negative orientation. Transcription of the GFP mini-replicon was assessed by GFP-expression. (figure 3). The plasmid from which the HK/68-R384G NP was expressed consistently resulted in fewer GFP positive cells than after transfection with wild type HK/68 NP or HK/68-E375G NP. Introduction of the co-mutation E375G in the HK/68-R384G gene increased the GFP expression in transfected cells, although the percentage of GFP positive cells was not as high as observed with the wild type HK/68 NP. Thus, the negative effect of R384G on viral fitness correlated with reduced functionality of the RNP-complex, which was partially overcome by the E375G co-mutation. Since the NP has multiple functions and interactions with viral and host cell proteins (199), its conformational integrity and functionality need to be retained for viral fitness. A similar finding was described recently for SIV escape from CTL recognition at a structurally constrained epitope in the gag protein (189).

Collectively, the data show that an amino acid substitution in a CTL epitope, which allows the virus to escape from the recognition by virus-specific CTL, is not tolerated because of functional constraints. Only when the R384G substitution occurs in the presence of other functionally compensating amino acid substitutions, this otherwise detrimental amino acid substitution is tolerated. We hypothesize that influenza viruses can exert sufficient flexibility to fix these mutations in the NP, although it should be realized that these events are relatively rare in comparison with the mutation rate in other proteins like the hemagglutinin. It also may explain that in some instances even immunodominant CTL epitopes like the HLA-A*0201-restricted $M1_{58-66}$ epitope from the matrix protein are fully conserved. For escape from specific CTL, additional substitutions may be required to overcome functional constraints imposed by the amino acid sequence of this epitope.

Acknowledgements

This work was supported by European Union grant QLRT-2001-01034 (Novaflu). Dr. R.A.M. Fouchier is a fellow of the Royal Dutch Academy of Arts and Sciences.

Chapter

4

Full restoration of viral fitness by multiple compensatory co-mutations in the nucleoprotein of influenza A virus cytotoxic T-lymphocyte escape mutants

G.F. Rimmelzwaan • E.G.M. Berkhoff • N.J. Nieuwkoop • D.J. Smith
R.A.M. Fouchier • A.D.M.E. Osterhaus

Department of Virology and WHO National Influenza Center, Erasmus Medical Center and Department of Zoology, Cambridge University, Cambridge, UK

Journal of General Virology 2005; 86:1801-5

Amino acid substitutions have been identified in the influenza A virus nucleoprotein that are associated with escape from recognition by virus-specific cytotoxic T-lymphocytes. One of these is the arginine-to-glycine substitution at position 384 (R384G). This substitution alone, however, is detrimental to viral fitness, which is overcome in part by the functionally compensating co-mutation E375G. Here, the effect of four other co-mutations associated with R384G on viral fitness was investigated using plasmid-driven rescue of mutant viruses. While none of these alternative co-mutations alone functionally compensated for the detrimental effect of the R384G-substitution, the M239V-substitution improved viral fitness of viruses containing 375G and 384R. The nucleoprotein displays unexpected flexibility to overcome functional constraints imposed by CTL epitope sequences allowing influenza viruses to escape from specific CTL.

Viruses can exploit a variety of different strategies to escape from recognition by virus-specific cytotoxic T-lymphocytes (CTL), which play an important role in the control of viral infections (168, 277). The accumulation of amino acid substitutions in or adjacent to CTL epitopes is one such strategy (138, 183) and has been described for a variety of viruses including HIV-1, SIV (98), hepatitis C virus (41, 74, 264) and influenza viruses (16, 26, 88, 209, 211, 253). It is a general property of RNA virus quasi species to adapt to changing environments and select for successful variants that e.g. have increased resistance to antiviral agents, have altered interferon-inducing capacity, escape from antibodies, and indeed, also from virus-specific CTL (65, 66). Amino acid substitutions at the anchor residue of a CTL epitope may prevent binding of the epitope to its corresponding MHC class I-molecule resulting in the loss of the epitope. Variation in T-cell receptor contact residues may prevent recognition by specific T-cell receptors present on virus-specific T-cells. Variation in CTL epitopes can thus result in effective immune evasion from CTL and has been shown to play an important role in the pathogenesis of various virus infections. The selective pressure exerted by CTL must be significant since it has been demonstrated that escape can occur at the cost of viral fitness. This was shown recently for HIV escape mutants, which reverted upon transmission to new susceptible hosts in which HIV CTL immunity is not present (83, 153). In addition, it has been demonstrated that in some cases viruses need to accumulate amino acid substitutions outside the epitope in order to restore viral fitness as a result of functional constraints imposed by the amino acid sequence in the epitope (83, 84, 133, 153, 189). Apparently viruses can overcome these functional constraints because there are examples of virus variants that escape from CTL but retain their viral fitness (84, 189). Amino acid substitutions in the influenza virus nucleoprotein have been described associated with escape from virus-specific CTL (16, 26, 88, 209, 211, 253), and these mutations can become fixed rapidly even when there are only small selective advantages—potentially due to population-dynamic effects (88). The R384G mutation in the HLA-B*2705 and -B*0801-restricted CTL epitopes

$NP_{383-391}$ and $NP_{380-388}$ respectively, resulted in a marked reduction of the human influenza virus-specific CTL response *in vitro* (16, 88, 209, 253). Thus, the immunodominant nature of the epitope (25), prolonged viral shedding in the absence of these epitopes in HLA-B*2705 and HLA-B*0801 positive individuals and population dynamics could have contributed to the emergence of these CTL escape variants. Also for these epitopes it recently has been demonstrated that an extra-epitopic co-mutation was required to functionally compensate the detrimental effect of the R384G substitution. It was shown that the naturally occurring E to G substitution at position 375 restored viral fitness partially in viruses in which the detrimental R384G mutation was introduced by site-directed mutagenesis. Position 384 is at the anchor residue of the epitopes and loss of the arginine resulted in loss of the anchor residue for binding to HLA-B*2705 and HLA-B*0801 (211, 253). Since the co-mutation at position 375 only partially compensated for the loss of viral fitness, and multiple co-mutations can be identified associated with the mutation at position 384 (58, 158), we wished to study the contribution of these additional mutations (R65K, D127E, I197V and M239V) to viral fitness in viruses that had escaped from $NP_{383-391}$ and $NP_{380-388}$ specific CTL.

To this end, recombinant influenza viruses were generated essentially as described previously (58, 196), using reverse genetics technology. For the generation of recombinant influenza viruses with amino acid substitutions at selected positions, the NP gene-segment of influenza virus A/Hong Kong/2/68 (A/HK/68) was amplified by RT-PCR as described previously (209) and inserted between the human pol I promotor and the hepatitis delta ribozyme sequence of plasmid pSP72-PhuThep (58). Site-directed mutagenesis of the NP gene was performed by PCR using the multi site-directed mutagenesis kit (Stratagene) according to the manufactures recommendations. The plasmids pHMG-NP, pHMG-PB1, pHMG-PB2, and pHMG-PA encoding the NP and the polymerase proteins PB1, PB2 and PA of influenza virus A/PR/8/34 respectively, were kindly provided by Dr. P. Palese (196). The bi-directional reverse genetics plasmids pHW181 through pHW188 encoding the viral gene segments of influenza virus A/WSN/33 were kindly provided by Dr. R.G. Webster (114). For the generation of recombinant viruses, the NP constructs of A/HK/68 were transfected into 293T cells together with pHMG-NP and the remaining genomic constructs of A/WSN/33 as described (58). Twenty-four hours after transfection, virus was passaged in Madin-Darby-Canine-Kidney (MDCK) cells and the infectious virus titers were determined as described (208). The combinations of mutations that were tested are listed in table 1. As shown in figure 1, of the single mutants only the R384G substitution was detrimental to viral fitness. Each of the other amino acid substitutions that were associated with R384G mutation had very modest effects on the virus titers compared to viruses containing the wild type sequence. As we described previously (209), the addition of the E375G substitution to the R384G substitution partially restored the viral replication, which was confirmed in the present study. In contrast, none of the other co-mutations functionally compensated the detrimental effect of the R384G substitution on viral fitness. This indicated that indeed E375G is of crucial impor-

Table 1. Plasmid constructs and their amino acid substitutions compared to the wild type A/HK/2/68 amino acid sequence

Plasmid	Amino acid position					
construct	384	375	239	197	127	65
WT	R	E	M	I	D	R
1	G					
2		G				
3			V			
4				V		
5					E	
6						K
7	G	G				
8	G		V			
9	G			V		
10	G				E	
11	G					K
12	G	G	V			
13	G	G		V		
14	G	G			E	
15	G	G				K

tance for viruses with the R384G mutation. Next we tested whether the other co-mutations played a role in viral fitness of viruses with the double mutation E375G/R384G. As shown in figure 1e-f, addition of the M239V mutation increased virus titers to the level of wild type virus. Thus, viruses containing 384G in combination with 239V and 375G, replicated to similar titers as wild type virus. Addition of the co-mutation D127E had no effect whilst R65K and I197V negatively influenced the virus replication of viruses with 375G/384G. These data suggest that position 239, 375 and 384 are involved in the same function of NP. Surprisingly however, 239V is not found in all viruses with 375E and 384G, for which sequences are available in the influenza sequence database at http://www.flu.lanl.gov/ (58, 158). We speculate that these viruses without 239V exist as intermediates. The effect of the respective mutations on viral fitness, exemplified in figure 1G, was confirmed by performing multi-step growth kinetics of these viruses after infection of MDCK cells by using an equivalent multiplicity of infection (MOI) of 0.001 50% tissue culture infectious dose per cell. Introduction of each of the single mutations (with the exception of R384G) did not alter the replication kinetics dramatically (figure 1H) compared to the replication of wild type virus. In figure 1I, the replication of viruses containing three amino acid substitutions were compared with wild type viruses and the E375G/R384G double mutant. In agreement with the data presented in figure 1E and F, introduction of 127E in the 375G/384G double mutant was neutral, whereas 65K and 197V had a negative effect on replication rate and 239V increased the replication rate to wild type level.

The replication rates of the respective viruses correlated with the functionality of the NP variants as measured in a NP transcomplementation assay that was performed as described previously (253). In brief, the NP-encoding sequence of influenza virus A/HK/2/68,

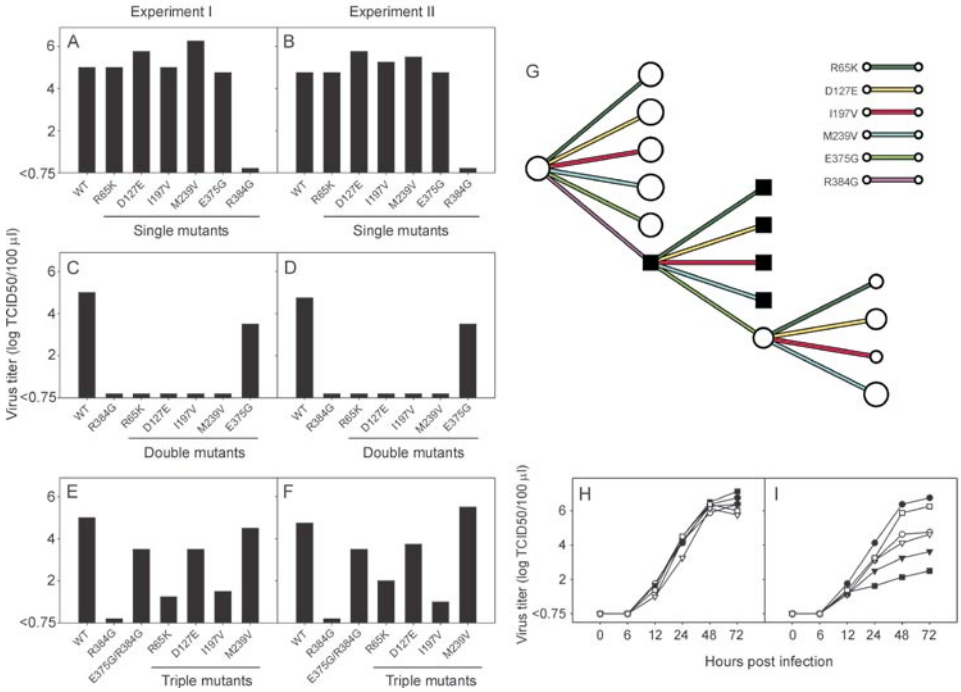


Figure 1. Amino acid substitutions E375G and M239V restore the detrimental effect on viral fitness of the R384G substitution in virus containing the NP sequence of influenza virus A/HK/2/68. In panels A and B, the infectious virus titers are shown obtained after infection of MDCK with viruses containing single amino acid substitutions (single mutants) or wild type (WT) virus, as indicated. In panels C and D, the results are shown obtained with viruses containing the R384G mutation in combination with one of the other amino acid substitutions as indicated (double mutants) or the R384G substitution only and WT. In panels E and F, the results are shown for viruses containing the E375G/R384G double mutation in combination with the other amino acid substitutions (triple mutants) or without and WT. The data of two independent experiments are shown. The tree (G) displays all mutants that were generated and shows the effect of the respective mutations on viral fitness of the recombinant influenza viruses. The circle area is proportional to the average viral titer obtained in the two experiments. Detrimental mutations preventing rescue of virus are indicated by solid squares. The mutations are indicated by line-color, R65K (dark green), D127E (yellow), I197V (red), M239V (light blue), E375G (light green), R384G (pink). Replication kinetics of NP mutant influenza A viruses were determined by performing multi step growth curves (H and I) of influenza viruses with single amino acid substitutions E375G (○), R65K (▼), D127E (▽), I197V (■), M239V (□), or without an amino acid substitution (wild type, ●) (H) or combinations of amino acid substitutions R384G/E375G without (○) or with R65K (▼), D127E (▽), I197V (■) or M239V (□) or without any substitution (wild type, ●) (I), after infection of MDCK cells at a MOI of 0.001. The data represent the average of two experiments.

with or without selected mutations was cloned into the eukaryotic expression plasmid pcDNA3. These plasmids were transfected into 293T cells along with pHMG-PB1, pHMG-PB2 and pHMG-PA and RF419, from which the green fluorescent protein (GFP) gene flanked by the non-coding region of the NS gene segment is transcribed in an anti-sense orientation. Transcription of the GFP mini replicon was assessed by GFP expression. As shown in figure 2, introduction of the E375G or the M239V substitutions by themselves did not have a significant effect on GFP expression in 293T cells. The R384G substitution reduced the functionality of the NP molecule markedly as was shown previously (209). The addition of

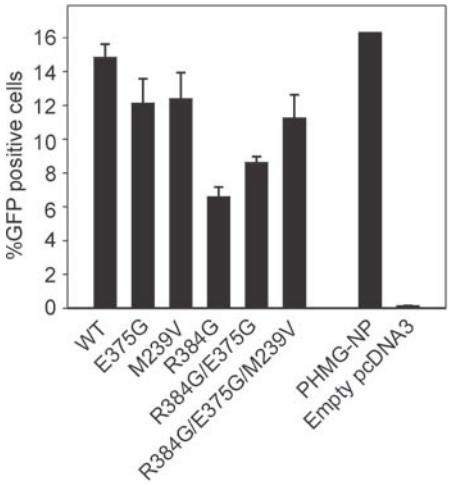


Figure 2. Functional analysis of A/HK/68 NP with or without various amino acid substitutions or combinations thereof, as indicated. Functionality of the NP was tested in a minireplicon reporter assay. 293T cells were transfected with plasmids pHMG-PB1, pHMG-PB2, pHMG-PA and RF419 and two µg plasmid encoding the NP of A/HK/68 with or without amino acid substitutions as indicated. Cells transfected with empty pcDNA3 served as negative controls, while cells cotransfected with pHMG-NP were used as positive control. The results are shown as the percentage of cells showing GFP expression, as measured by flow-cytometry. The results represent the average of two independent experiments that were carried out in duplicate. The error bars indicate one standard deviation. With the plasmid pEGFP-N1 transfection efficiencies were typically >90%.

E375G partially restored NP functionality, which was further improved by the introduction of the M239V substitution (Figure 2). The number of GFP^+ cells obtained with this triple mutant was comparable to that obtained with the wild type NP sequence, confirming that multiple co-mutations are required for full restoration of NP functionality. Although there was a correlation between GFP expression and replication rates, the NP with the R384G substitution showed residual transcriptional activity in the absence of demonstrable virus replication. It is unclear how much of this activity is required for virus replication. Apparently, there is no simple linear correlation between the two. Alternatively, other functional properties of the NP were affected by the R384G substitution that are of importance at other stages of the virus replication cycle. The NP has many different functions and can interact with a variety of viral and cellular proteins (199). Despite the many functional constraints on the protein, it also shows a remarkable flexibility.

Our data show that the introduction of an otherwise detrimental amino acid substitution, which allows the virus to escape from virus-specific cytotoxic T-lymphocytes (16, 88, 209, 253) can be compensated for by the accumulation of a number of different co-mutations. Similar functionally compensating mutations have been observed in HIV and SIV CTL escape mutants (83, 84, 133, 153, 189), indicating that this is a common strategy of RNA viruses. In contrast to the lentiviruses, which cause chronic infections in their hosts, the selective pressure against influenza virus CTL epitopes most likely is exerted through

herd immunity in the human population, induced by annual epidemics in a significant proportion of the population (88).

Acknowledgements

This work was supported by European Union grant QLRT-2001-01034 (Novaflu). R.A.M.F is a fellow of the Royal Dutch Academy of Arts and Sciences.

Chapter

5

Functional constraints of influenza A virus epitopes limit escape from cytotoxic T-lymphocytes

E.G.M. Berkhoff • E. de Wit • M.M. Geelhoed-Mieras • A.C.M. Boon
J. Symons • R.A.M. Fouchier • A.D.M.E. Osterhaus • G.F. Rimmelzwaan

Department of Virology and WHO National Influenza Center,
Erasmus Medical Center, Rotterdam, The Netherlands

Journal of Virology 2005; 79:11239-46

Viruses can exploit a variety of strategies to evade immune surveillance by cytotoxic T-lymphocytes (CTL), including the acquisition of mutations in CTL epitopes. Also for influenza A viruses, a number of amino acid substitutions in the nucleoprotein (NP) have been associated with escape from CTL. However, other previously identified influenza A virus CTL epitopes are highly conserved, including the immunodominant HLA-A*0201-restricted epitope from the matrix protein, $M1_{58-66}$. We hypothesized that functional constraints were responsible for the conserved nature of influenza A virus CTL epitopes, limiting escape from CTL. To assess the impact of amino acid substitutions in conserved epitopes on viral fitness and recognition by specific CTL, we performed a mutational analysis of CTL epitopes. Both alanine-replacements and more conservative substitutions were introduced at various positions of different influenza A virus CTL epitopes. Alanine-replacements for each of the nine amino acids of the $M1_{58-66}$ epitope were tolerated to variable extents, except for the anchor residue at the second position. Substitution of anchor residues, in other influenza A virus CTL epitopes also affected viral fitness. Viable mutant viruses were used in CTL recognition experiments. The results are discussed in the light of the possibility of influenza viruses to escape from specific CTL. It was speculated that functional constraints limit variation in certain epitopes, especially at anchor residues, explaining the conserved nature of these epitopes.

Introduction

Cytotoxic T-lymphocytes (CTL) play an important role in the control of viral infections (60). To evade these CTL responses, viruses can exploit a variety of mechanisms to prevent recognition by specific CTL, including the accumulation of amino acid substitutions in or adjacent to CTL epitopes (138, 183). This strategy has been shown predominantly by certain RNA viruses, such as human immunodeficiency virus (HIV) (29, 96, 97, 170, 191, 200, 201), hepatitis C virus (41, 264) and lymphocytic choriomeningitis virus (171, 195), which are known for their high mutation rate. Also for influenza A viruses, a number of amino acid substitutions in the nucleoprotein (NP) have been associated with escape from human CTL. One of them, the R to G substitution at position 384 (R384G), which is at the anchor residues of the HLA-B*0801-restricted $NP_{380-388}$ and HLA-B*2705-restricted $NP_{383-391}$ epitopes resulted in the loss of these epitopes (211, 253). This substitution reduced the *in vitro* virus-specific CTL response in HLA-B*2705 positive individuals significantly (16). Although the R384G substitution was tolerated only in the presence of one or more functionally compensating co-mutations (209, 210), it was fixed rapidly. This was explained by small selective advantages and population dynamics in a theoretical model (88). A third variable epitope in the influenza A virus NP is the HLA-B*3501-restricted epitope $NP_{418-426}$, which displayed considerable variability in T-cell contact residues, affecting recognition by

specific CTL (26, 27). Thus, in contrast to the two epitopes described above, the $NP_{418-426}$ epitope retained its anchor residues for binding to HLA-B*3501. Other previously identified epitopes in influenza A virus proteins are highly conserved, such as the immunodominant HLA-A*0201-restricted epitope from the matrix protein, $M1_{58-66}$. It is likely that selective pressure by CTL against this epitope is high, considering the immunodominant nature of the epitope (25, 231) and the high prevalence of HLA-A*0201 in the human population (163). Yet, the amino acid sequence of this nine-mer epitope is conserved, even between different subtypes of human influenza A virus. We hypothesize that functional constraints are responsible for the virus' inability to accumulate amino acid substitutions in this and other conserved epitopes, limiting immune escape from virus-specific CTL responses. To test this hypothesis, we performed a mutational analysis of various epitopes and tested the effect of selected amino acid substitutions on viral fitness and immune recognition by CTL. For this purpose, we employed a plasmid-driven rescue system for the generation of recombinant influenza viruses. For the epitope $M1_{58-66}$ (GILGFVFTL), we performed alanine-replacements for each of the nine amino acid positions. In addition, various other amino acid substitutions were introduced in this and four other epitopes, namely the HLA-A*0101-restricted epitopes $PB1_{591-599}$ and NP_{44-52}, the HLA-B*2705-restricted epitope $NP_{174-184}$, and the HLA-B*3501-restricted epitope $NP_{418-426}$. Single mutations at anchor residues could result in the loss of the epitopes, which would constitute the most economic way for the virus to escape from immune surveillance by specific CTL. Therefore, we focused on the mutational analysis of anchor residues of the respective epitopes. The data obtained in the present study on viral fitness and recognition of influenza viruses with mutations in CTL epitopes are discussed in the light of natural evolution of CTL epitopes.

Material and methods

Plasmids

For the generation of recombinant influenza viruses, a bidirectional reverse genetics system based on influenza virus A/Netherlands/178/95 (A/NL/178/95; H3N2), was used. The viral gene segments were amplified by RT-PCR using segment-specific primers, purified by electroforesis in agarose gels according to standard methods, and cloned into a modified pHW2000 vector as previously described (58, 114). Subsequently, site-directed mutagenesis was performed (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA) to substitute single amino acids in several influenza virus CTL epitopes, as listed in table 1. Sequence analysis was performed for all recombinant plasmids, using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), according to the instructions of the manufacturer. All PCR primer sequences and plasmid maps are available on request.

Table 1. Substitutions introduced in CTL epitopes as indicated

Epitope	Amino acid sequence											Substitution	Rescued
$M1_{58-66}$	G	I	L	G	F	V	F	T	L			WT	Yes
	A											G58A	Yes
		A										I59A	No
			A									L60A	Yes
				A								G61A	Yes
					A							F62A	Yes
						A						V63A	Yes
							A					F64A	Yes
								A				T65A	Yes
									A			L66A	Yes
		L										I59L	Yes
		V										I59V	Yes
$NP_{418-426}$	L	P	F	E	K	S	T	V	M			WT	Yes
		A										P419A	No
		G										P419G	No
									A			M426A	No
									I			M426I	Yes
$PB1_{591-599}$	V	S	D	G	G	P	N	L	Y			WT	Yes
			N									D593N	No
NP_{44-52}	C	T	E	L	K	L	S	D	Y			WT	Yes
			Q									E46Q	Yes
$NP_{174-184}$	R	R	S	G	A	A	G	A	A	V	K	WT	Yes
		K										R175K	Yes

Generation of viruses

The recombinant bidirectional plasmids were transfected into 293T cells, using the calcium phosphate precipitation method as described previously (58). After 48 hours, culture supernatants were harvested, and used for subsequent infection of confluent Madin-Darby Canine Kidney (MDCK) cells (16). After three days, culture supernatants were harvested, cleared by low speed centrifugation, aliquoted and stored at -80 °C until use. The recombinant viruses were designated A/NL/95- and their specific substitutions. In order to confirm the introduction of the mutations and to exclude the introduction of second site mutations the nucleotide sequence of the corresponding full-length genes were assessed. Infectious virus titers were determined as previously described (208). Multi-step growth kinetics were performed for all recombinant influenza viruses after infection of MDCK cells, using an equiv-

alent multiplicity of infection (MOI) of 0.001 50% tissue culture infectious dose ($TCID_{50}$) per cell, which was used as a measure of viral fitness. Viral fitness was considered reduced when a statistically significant difference was observed compared to plasmid-derived wild type virus.

$CD8^+$ T-cell clones

Generation of $CD8^+$ T-cell clones directed against the HLA-A*0201-restricted epitope $M1_{58-66}$, the HLA-B*3501-restricted epitope $NP_{418-426}$, the HLA-A*0101-restricted epitopes NP_{44-52} and $PB1_{591-599}$ was described previously (27, 253).

Target cells

B-lymphoblastoid cell lines (BLCL), established as described previously (212), and three C1R cell lines, kindly provided by Dr. P. Romero (HLA-A*0201-transfected C1R cell line), Dr. M. Takiguchi (HLA-B*3501-transfected C1R cell line) and Dr. P. Cresswell (HLA-A*0101-transfected C1R cell line), were used as target cells. Peptide labeling was performed by incubating 10^6 cells/ml overnight with 5 μM peptide in RPMI 1640 medium (Cambrex, East Rutherford, NJ, USA) supplemented with 10% FCS and antibiotics (R10F). Peptides were manufactured, HPLC-purified (immunograde, >85% purity) and analyzed with mass spectrometry (Eurogentec, Seraing, Belgium). For infection with the recombinant influenza viruses, 10^6 target cells were infected at a MOI of 3 in a volume of 1 ml. After incubation for one hour at 37 ºC the cells were resuspended in R10F and incubated for 16-18 hrs.

Intracellular IFN-γ staining and flow cytometry

The $CD8^+$ T-cell clones were adjusted to a concentration of 10^6 cells/ml in R10F supplemented with Golgistop (monensin; Pharmingen, Alphen a/d Rijn, The Netherlands). Sixty thousand effector cells were incubated with 3×10^5 stimulator cells, which were infected, pulsed with peptides or left untreated, for 6 hours at 37 ºC in U-bottom plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands). Subsequently intracellular IFN-γ-staining was performed as described previously (28). In brief, the cells were washed with PBS containing 2% FCS (P2F) and Golgistop, stained with monoclonal antibody (MAb) directed to CD3 (Becton Dickinson, Alphen a/d Rijn, The Netherlands) and CD8 (Dako, Glostrup, Denmark), fixed and permeabilized with cytofix and cytoperm (Pharmingen) and stained with a MAb specific for IFN-γ (Pharmingen). At least 5×10^3 gated $CD3^+$ $CD8^+$ events were acquired using a FACSCalibur (Becton Dickinson) flowcytometer. The data were analyzed using the software program Cell Quest Pro (Becton Dickinson).

ELIspot

ELIspot assays were performed as described previously (25). In brief, 96-well Silent Screen Plates (Nalge Nunc International, Rochester, NY, USA) were coated with 2.5μg/ml of anti-IFN-γ MAb 1-DIK (Mabtech, Stockholm, Sweden), and blocked with RPMI 1640 medium supplemented with 10% human AB serum (Sanquin Bloodbank, Rotterdam, The Netherlands), antibiotics and 20μM β-mercaptoethanol (R10H). Three thousand cells of $CD8^+$ T-cell clones were incubated with 3×10^4 target cells, which were infected, pulsed with peptides or left untreated, for 4 hours. Next, the plates were washed with PBS-0.005% Tween-20 (Sigma Chemical Co., St. Louis, MO, USA), and secreted IFN-γ was detected using biotinylated anti-IFN-γ MAb 7-B6-1 (Mabtech; dilution of 1:5000). Subsequently, streptavidin labelled with alkalin phosphatase was added, which was visualized with phosphatase substrate BCIP/NBT (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Numbers of spots were counted using an automated image analysis ELIspot reader (Aelvis, Sanquin Bloodbank, Amsterdam, The Netherlands).

Chromium-release assay

Chromium-release assays were performed as described previously (25). In brief, 7.5×10^5 cells per target were labelled with 75 μCi $Na_2[^{51}Cr]O_4$, and incubated with $CD8^+$ T-cell clone at effector-to-target (E:T) ratios of 10, 5, 2.5, and 1. Target cells were also incubated with 10% Triton X-100 or R10F to determine the maximum and spontaneous release, respectively. After 4 hrs incubation, the supernatants were harvested (Skatron instruments, Sterling, VA, USA) and radioactivity was measured by gamma-counting. The percentage specific lysis was calculated with the following formula: ((experimental release-spontaneous release) / (maximum release-spontaneous release)) × 100%. The chromium-release assays were performed in quadruplicate and the data were presented as the average.

Synonymous/Non-synonymous Analysis

The ratio of synonymous and non-synonymous nucleotide substitutions was calculated, using a synonymous/non-synonymous analysis program (SNAP) (137, 178, 184) at www.hiv.lanl.gov. The NP nucleotide sequences of influenza viruses A/England/878/69 (AY210221) and A/New York/12/2003 (CY000124) obtained from the Influenza Sequence Database (www.flu.lanl.gov) (158) were used in comparison for analysis by SNAP. These viruses were selected since the NP genes belonged to the same lineage of influenza A (H3N2) viruses (154).

Results

Synonymous/Non-synonymous Analysis

In order to obtain an impression of the selective pressure mediated by virus-specific CTL, we performed a synonymous/non-synonymous analysis with the NP nucleotide sequences of a pair of influenza A (H3N2) viruses consisting of influenza virus A/England/878/69, isolated shortly after the introduction of H3N2 virus in the human population, and a more recent strain, A/New York/12/2003. The NP gene was selected for this type of analysis since fourteen of the known epitopes are located within this protein. The synonymous/non-synonymous (ds/dn) ratio for the sequence encoding the fourteen epitopes was 8.67, whereas the ds/dn ratio for the rest of the protein was 19.73, which is suggestive for selective pressure on the CTL epitopes. However, blast-search of up to 450 influenza A H3N2 virus sequences indicated that all known CTL epitopes, including those located within other viral proteins, retained their anchor residues with the exception of the HLA-B*0801 and HLA-B*2705-restricted epitopes $NP_{380-388}$ and $NP_{383-391}$ (see discussion section).

Viral fitness of influenza viruses with mutations in CTL epitopes

Since the HLA-A*0201-restricted epitope $M1_{58-66}$ (GILGFVFTL) is highly conserved, we selected this epitope to examine the effect of alanine-replacements at each of the nine positions of the epitope on viral fitness. Mutant viruses could be rescued with alanine-replacements at all positions within the $M1_{58-66}$ epitope, except for the second position (figure 1A). The alanine-replacement at position 59 of the matrix protein, which is the anchor residue of the $M1_{58-66}$ epitope, was detrimental to viral fitness. Although viruses with alanine-replacements at the other eight positions were rescued, the virus replication kinetics of these mutants was affected compared to wild type virus (figure 1B). Especially, mutant viruses A/NL/95-M1 F62A and -M1 F64A yielded >100-fold less progeny virus than the wild type virus at twelve hours post infection. At 48 hours post infection the differences with wild type virus were still at least 50-fold. In addition, the L60A substitution caused a reduction of 75-fold in virus production compared to wild type virus from 24 hours onwards. Since the dramatic effect of the alanine-replacement at the anchor residue was of special interest, we decided to study the effect of more conservative substitutions at position 59. We also replaced the isoleucin at this position for a leucine and a valine (M1 I59L and I59V) and found that in contrast to M1 I59A these replacements were tolerated by the virus to a certain extent, since these mutant viruses were readily rescued (figure 1C). We also performed multi-step growth curves with these mutant viruses. Six and twelve hours post infection these mutant viruses yielded 100-fold and 30-fold less progeny virus than wild type virus respectively. From 24 hours post infection onwards these differences were no longer statistically significant (figure 1D).

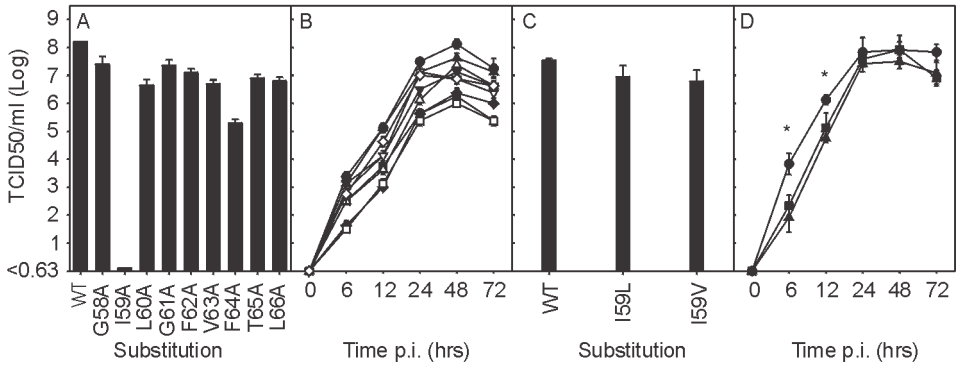


Figure 1. Effect of amino acid substitutions in the $M1_{58-66}$ epitope on viral fitness. Upon transfection of 293T cells and subsequent rescue in MDCK cells, infectious virus titers were determined for the wild type and mutant influenza viruses with alanine-replacements for each of the nine amino acids of the $M1_{58-66}$ epitope (A) and with the more conservative substitutions at position 59 (C). Influenza virus could be rescued with alanine-replacements at all positions within the $M1_{58-66}$ epitope, except for position 59 (A). Influenza A virus tolerated the more conservative substitutions M1 I59L and I59V to a certain extent (C). The data represent the average of three experiments. Subsequently, growth curves were generated (B and D) after infection of MDCK cells at an MOI of 0.001. Virus replication kinetics for wild type virus (●) and M1 G58A (▲), L60A (■), G61A (▼), F62A (♦), V63A (Δ), F64A (□), T65A (∇), L66A (◊) (B), and wild type virus (●), I59L (▲) and I59V (■) (D) are shown. The data represent the average of three experiments. The error bars indicate the standard deviation. Asterisk (*) indicates statistical significance ($p<0.05$; student t-test).

Because of the impact of the alanine-replacement at the anchor residue of the $M1_{58-66}$ epitope, we decided to study the effect on anchor residue substitutions of the HLA-B*3501-restricted epitope $NP_{418-426}$ (LPFEKSTVM). This epitope displays a high degree of variability, but retained its anchor residues for binding to HLA-B*3501 (26). Replacement of the proline at position 419 or the methionine at position 426 for an alanine at these positions, were detrimental to viral fitness (figure 2A). A more conservative substitution at position 419 (NP P419G) also prevented rescue of viable virus. The conservative NP M426I substitution was not detrimental to viral fitness, and only six hours post infection virus replication kinetics were significantly impaired (38-fold) compared to wild type virus (figure 2A and B).

In addition, conservative amino acid substitutions at the anchor residues of the HLA-A*0101-restricted epitopes $PB1_{591-599}$ (VSDGGPNLY) and NP_{44-52} (CTELKLSDY), and the HLA-B*2705-restricted epitope $NP_{174-184}$ (RRSGAAGAAVK) were introduced. The D593N substitution in PB1 was detrimental to viral fitness. The E46Q and R175K substitutions in the viral NP were not detrimental to viral fitness (figure 3A), although mutant virus A/NL/95-NP E46Q yielded up to 133-fold less progeny virus within the first 24 hours post infection than wild type virus. For mutant virus A/NL/95-NP R175K significantly lower virus titers were observed than for wild type virus from 48 hours post infection onwards (figure 3B).

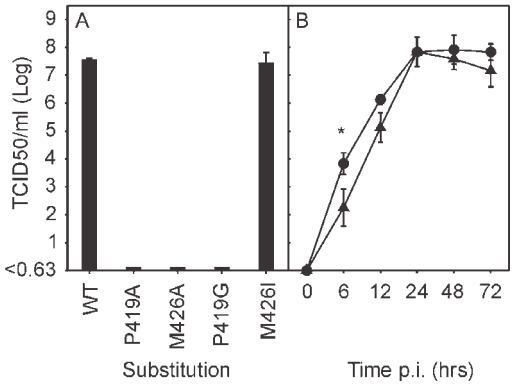


Figure 2. Effect of amino acid substitutions in the $NP_{418-426}$ epitope on viral fitness. Upon transfection of 293T cells and subsequent rescue in MDCK cells, infectious virus titers were determined for the wild type and mutant influenza viruses with alanine-replacements at position 419 or 426, or with the more conservative substitutions NP P419G and M426I (A). The data represent the average of three experiments. Subsequently, growth curves of wild type virus (●) and influenza virus A/NL/95-NP M426I (▲) were generated after infection of MDCK cells at an MOI of 0.001 (B). The data represent the average of three experiments. The error bars indicate the standard deviation. Asterisk (*) indicates statistical significance ($p<0.05$; student t-test).

Recognition of mutant viruses by CTL

The recognition of HLA-A*0201 positive cells infected with the various $M1_{58-66}$ mutant viruses by specific CTL was determined by intracellular IFN-γ staining, ELIspot assay and classical chromium release assays. As shown in figure 4, the $M1_{58-66}$ specific CTL clone recognized C1R-A2 cells infected with wild type virus and mutant A/NL/95 virus with the M1 G58A, L60A or L66A substitution, but not cells infected with A/NL/95 mutant viruses with the M1 G61A, F62A, V63A or T65A substitution or non-infected cells as determined by intracellular IFN-γ staining and flow cytometry. These observations were confirmed by ELIspot (figure 4K and L) and by chromium release assays (figure 4M and N). A control CTL clone specific for the $NP_{418-426}$ epitope recognized target cells infected with all mutant A/NL/95 viruses similarly, indicating that the infection of the cells, the processing and presentation of immunogenic peptides was comparable for all viruses (figures 4L and N). The recognition of A/NL/95-M1 I59A and -F64A could not be tested, since these mutant viruses could not be propagated to sufficiently high titers. C1R-A2 cells infected with A/NL/95 mutant viruses with the more conservative amino acid substitutions M1 I59V and I59L were fully recognized in all three assays (figure 5).

Of four mutants tested, mutant virus A/NL/95-NP M426I was the only virus with an amino acid substitution in the $NP_{418-426}$ epitope that proved viable and with which CTL recognition was studied. HLA-B*3501 and -A*0201 positive cells infected with wild type virus were recognized by $M1_{58-66}$ specific CTL and $NP_{418-426}$ specific CTL by intracellular IFN-γ staining, ELIspot and in chromium release assays (figure 6B, E and G). However, cells infected with A/NL/95-NP M426I were recognized by $M1_{58-66}$ specific CTL, but not by $NP_{418-426}$ specific CTL (figure 6C, F and H). These results were confirmed by showing that

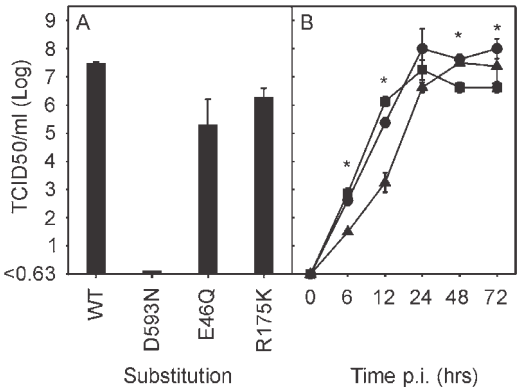


Figure 3. Effect of amino acid substitutions on viral fitness. Upon transfection of 293T cells and subsequent rescue in MDCK cells, infectious virus titers were determined for the wild type and mutant influenza viruses with the conservative amino acid substitutions PB1 D593N, NP E46Q or NP R175K in epitopes $PB1_{591-599}$, NP_{44-52} and $NP_{174-184}$ respectively (A). The data represent the average of three experiments. Subsequently, growth curves of wild type virus (●) and mutant viruses A/NL/95-NP E46Q (▲) and -R175K (■) were generated after infection of MDCK cells at a MOI of 0.001 (B). The data represent the average of three experiments. The error bars indicate the standard deviation. Asterisk (*) indicates statistical significance between wild type and influenza virus A/NL/95-NP E46Q at 6 and 12 hours post-infection, and between wild type and influenza virus A/NL/95-NP R175K at 48 and 72 hours post-infection ($p<0.05$; student t-test).

the functional avidity of the $NP_{418-426}$ specific CTL decreased more than 100-fold by the NP M426I substitution, using serial dilutions of wild type and mutant peptide in ELIspot assays (data not shown).

The recognition of influenza virus A/NL/95-PB1 D593N was not tested, since this mutant virus could not be rescued. Although influenza virus A/NL/95-NP E46Q could not be propagated to sufficiently high titers, the recognition of HLA-A*0101 positive cells infected at a low MOI (0.02) was examined by ELIspot. It was found that the substitution at the anchor residue abrogated recognition by NP_{44-52} specific CTL (data not shown). Influenza virus A/NL/95-NP R175K was not tested, since no specific CTL clone was available for this epitope.

Discussion

In the present paper the effect of amino acid substitutions in CTL epitopes on viral fitness and T-cell recognition was evaluated. It was concluded that functional constraints imposed on CTL epitopes limit escape from virus-specific CTL without loss of viral fitness.

The synonymous/non-synonymous analysis revealed that in the 90 amino acids that constitute the fourteen known epitopes located in the NP, relatively more non-synonymous mutations occurred between 1969 and 2003 than in the rest of the protein. The hypervariable epitope $NP_{418-426}$ had a major impact on the lower ds/dn ratio and five out of the fourteen partially overlapping epitopes were fully conserved. Some points in this analysis should

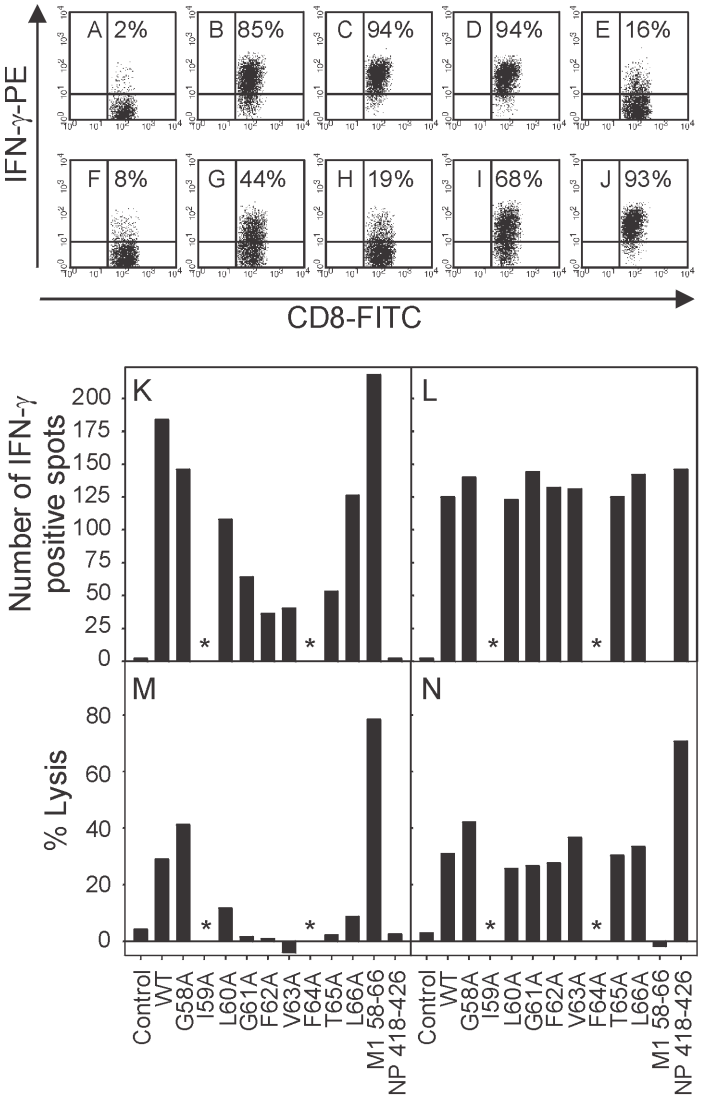


Figure 4. Effect of alanine-replacements in the $M1_{58-66}$ epitope on recognition by specific CTL. Reactivity of CTL clone, directed against the $M1_{58-66}$ epitope, with stimulator cells infected with wild type (B) or influenza virus A/NL/95-M1 G58A (C), -L60A (D), G61A (E), -F62A (F), -V63A (G), -T65A (H) or -L66A (I) was determined by intracellular IFN-γ staining and flow cytometry. GILGFVFTL-peptide pulsed cells were included as a positive control (J). Untreated cells as a negative control (A). Indicated is the percentage IFN-γ^+ cells within the $CD8^+$ T cell population. The data are also presented as the number of IFN-γ positive spots, as measured in an IFN-γ specific ELIspot (K). And as the percentage specific lysis, as measured in chromium release assays (M). Effector cells were added at an effector-to-target cell ratio of 10, and specific lysis was calculated. A CTL clone specific for the $NP_{418-426}$ epitope was used as a control (L, N). The recognition of A/NL/95-M1 I59A and -F64A could not be tested, since these mutant viruses could not be propagated to sufficiently high titers (*). The data of representative experiments is shown.

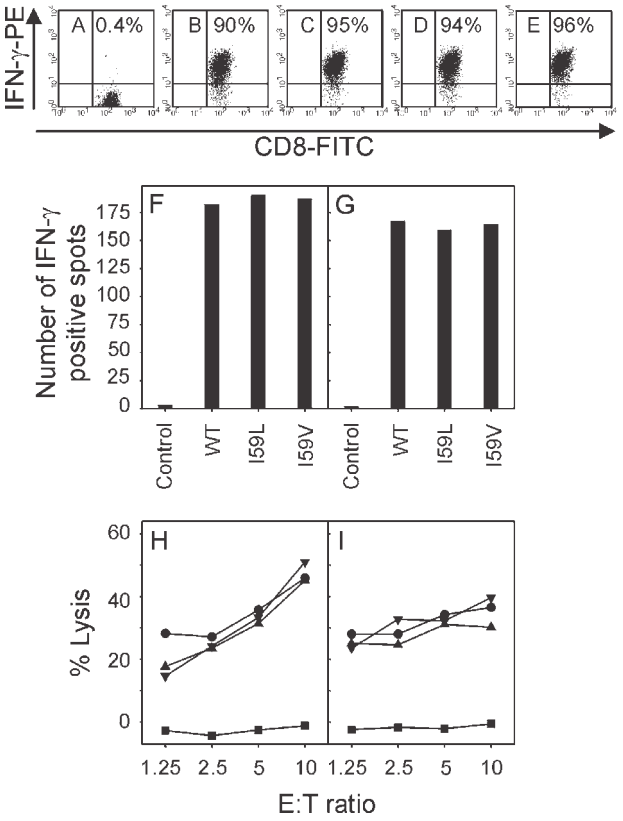


Figure 5. Effect of selected amino acid substitutions in the $M1_{58-66}$ epitope on recognition by specific CTL. Reactivity of CTL clone, directed against the $M1_{58-66}$ epitope, with stimulator cells infected with wild type (B) or influenza virus A/NL/95-M1 I59L (C) or -I59V (D) was determined by intracellular IFN-γ staining and flow cytometry. GILGFVFTL-peptide pulsed cells were included as a positive control (E). Untreated cells as a negative control (A). Indicated is the percentage IFN-γ^+ cells within the $CD8^+$ T cell population. The data are also presented as the number of IFN-γ positive spots, as measured in an IFN-γ specific ELIspot (F). And as the percentage specific lysis, as measured in chromium release assays (H). Effector cells were added at different effector-to-target cell ratios as indicated, and specific lysis was calculated. A CTL clone specific for the $NP_{418-426}$ epitope was used as a control (G, I). The data of representative experiments is shown.

be taken into consideration. First, since commonly old prototypic strains like A/Puerto Rico/8/34 have been used for the identification of influenza virus CTL epitopes, there is a bias towards the identification of conserved epitopes (61-63, 87, 125, 158, 160, 216, 245, 263). Recent work in our laboratory indicates that a significant number of epitopes is not conserved (Berkhoff *et al.* unpublished data). Second, the conserved epitopes and the variable epitopes, including the $NP_{418-426}$ epitope have in common that they all retained their anchor residues for binding to their corresponding HLA molecules. The only exception to this is an amino acid substitution at position 384 of the NP. The R384G substitution, which is at the anchor residues of the HLA-B*0801 and -B*2705-restricted epitopes $NP_{380-388}$ and $NP_{383-391}$, resulted in the loss of their epitopes and abrogated recognition of virus infected

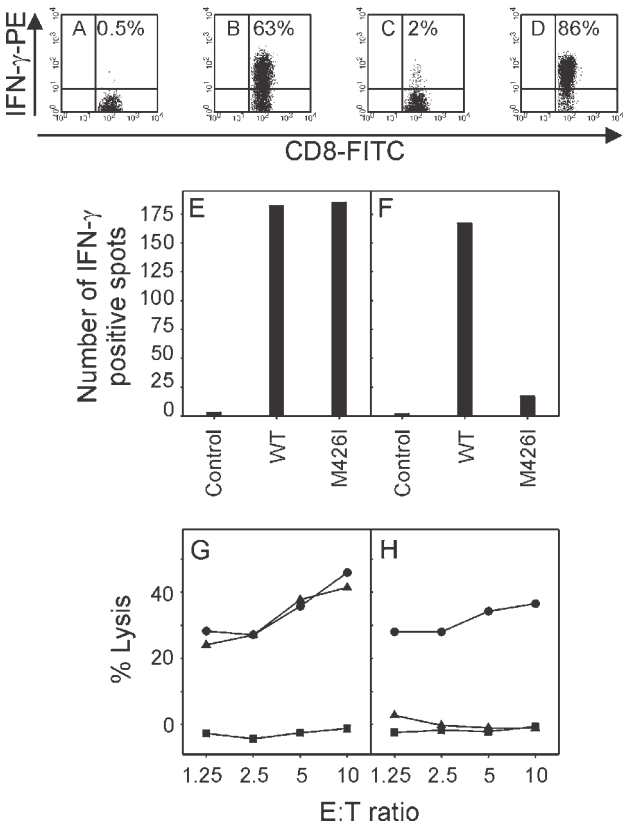


Figure 6. Amino acid substitution NP M426I affects recognition by specific CTL. Reactivity of CTL clone, directed against the $NP_{418-426}$ epitope, with stimulator cells infected with wild type (B) or influenza virus A/NL/95-NP M426I (C) was determined by intracellular IFN-γ staining and flow cytometry. LPFEKSTVM-peptide pulsed cells were included as a positive control (D). Untreated cells as a negative control (A). Indicated is the percentage IFN-γ^+ cells within the $CD8^+$ T cell population. The data are also presented as the number of IFN-γ positive spots, as measured in an IFN-γ specific ELIspot (F). And as the percentage specific lysis, as measured in chromium release assays (H). Effector cells were added at different effector-to-target cell ratios as indicated, and specific lysis was calculated. A CTL clone specific for the $M1_{58-66}$ epitope was used as a control (E, G). The recognition of A/NL/95-NP P419A, -M426A and -P419G could not be tested, since these mutations prevented rescue of viable virus. The data of representative experiments is shown.

cells by specific CTL (211, 253). However, introduction of a glycine at position 384 of the NP of influenza virus A/Hong Kong/2/68 was detrimental to viral fitness, and several co-mutations associated with the R384G substitution in epidemic influenza virus strains were required to functionally compensate the detrimental effect of the R384G substitution (209, 210). Similar findings have been observed for CTL escape mutants of HIV and SIV, which also accumulated extra-epitopic co-mutations in the gag-protein for restoration of viral fitness in the presence of mutations in CTL epitopes (84, 133, 189). Apparently, RNA viruses display sufficient flexibility to escape from CTL and retain viral fitness. For HIV and SIV the selective pressure is mediated by CTL during the chronic infection of individual hosts, while for influenza viruses this takes place by CTL immunity at the population level (88). It

is of special interest that also for HIV, CTL escape mutants can be identified at the population level (194), although transmission rates of this virus are much lower than for influenza viruses. Thus, influenza virus CTL epitopes are either conserved, display variation at non-anchor residues or lose their anchor residues at the cost of viral fitness, which is functionally compensated by the accumulation of co-mutations. To assess the impact of amino acid substitutions in conserved epitopes on viral fitness and recognition by specific CTL, we conducted a mutational analysis of the epitope $M1_{58-66}$ (GILGFVFTL). This epitope is immunodominant and recognized by a large portion of individuals in the population, but is highly conserved. Replacement of the anchor residue at position 2 of the epitope (M1 I59A) was detrimental to viral fitness, whereas alanine-replacements at the other eight positions did not prevent rescue of recombinant influenza virus and were tolerated to variable extents. The $M1_{58-66}$ epitope is located in the fourth N-terminal α-helix of the M1 protein. Mutations in this region may disturb the functional and structural integrity of the protein as has been described for mutations in the M1 helix six domain (35, 156). The reduced virus titers obtained with a number of these mutant M1 viruses correlated with the number of productively infected cells as measured by immuno-fluorescence assay using a NP-specific monoclonal antibody six hours post infection of MDCK cells, suggesting that the virus replication cycle was affected at an early pre-transcriptional stage (data not shown). Conservative amino acid substitutions at position 2 of the $M1_{58-66}$ epitope (M1 I59L and I59V) were less critical, although the kinetics of viral replication was somewhat affected. More importantly, the A/NL/95-M1 I59L and -I59V mutant viruses were fully recognized by $M1_{58-66}$ specific CTL, which makes it unlikely that these variants would ever emerge in the human population. Although some of the other alanine-replacements resulted in the partial loss of recognition by $M1_{58-66}$ specific CTL, their impaired replication kinetics is not in favor of the emergence of these mutants. We speculate that there must be a trade-off between viral fitness and immune recognition of which we have little insight at present. The T-cell recognition patterns that were observed here with mutant virus infected cells was in agreement with those observed with mutant $M1_{58-66}$-peptides in previous studies (10, 93, 186). Although the use of T-cell clones may not reflect the situation *in vivo*, the analysis of anchor residues boils down to recognition of the epitope or not, which is not different between clonal and polyclonal T-cell populations. In the analysis of T-cell receptor contact residues as done for the $M1_{58-66}$ epitope, the situation is more complicated. However, the $M1_{58-66}$ specific CTL response is oligoclonal in nature and dominated by T-cells carrying the TCR with Vβ17 chains (151, 172). Fitness costs also limit variation in the highly immunodominant Gag p11C, C-M CTL epitope of SIV and escape from specific CTL (190). Therefore, this phenomenon may be more universal and apply for more RNA viruses, which are under selective pressure mediated by CTL. It even may contribute to shaping of the T-cell repertoire and have an influence on the hierarchy of epitope dominance.

Next, we wished to evaluate the conservative anchor residues of the otherwise hypervariable epitope $NP_{418-426}$ (LPFEKSTVM). The relatively conservative NP P419A and P419G substitutions at position 2 of the epitope were both detrimental to viral fitness, indicating that the proline at this position is essential. Amino acid substitutions at position 9 of the epitope, the second anchor residue, yielded interesting results. First, the NP M426A substitution was detrimental to viral fitness. Second, with the conservative NP M426I substitution, the HLA-B*3501 binding motif was retained (75, 109, 234) and viral fitness was not affected to a great extent. Of special interest, HLA-B*3501 positive cells infected with influenza virus A/NL/95-NP M426I were poorly recognized by $NP_{418-426}$ specific T-cell clones. Since the NP M426I mutant epitope retained its capacity to bind to HLA-B*3501, it may have undergone conformational changes in T-cell receptor contact residues, preventing recognition by CTL as has been described previously for an other HLA-B*3501-restricted epitope (67). Conservative amino acid substitutions at the anchor residues of the epitopes $PB1_{591-599}$ (VSDGGPNLY), NP_{44-52} (CTELKLSDY) and $NP_{174-184}$ (RRSGAAGAAVK) also affected viral fitness. The PB1 D593N substitution in particular was detrimental to viral fitness. Although the conservative NP E46Q substitution resulted in the loss of the anchor residue and would allow the virus to escape from specific CTL, the loss of viral fitness may limit the emergence of this variant in the human population.

Based on the data presented here, we speculate that influenza A viruses display a limited degree of variation in CTL epitopes despite selective pressure on these epitopes mediated by CTL. Functional constraints imposed on influenza virus CTL epitopes may limit efficient escape from CTL and could constitute the Achilles heel of these viruses, limiting the impact of epidemic and pandemic outbreaks of influenza on severe morbidity and mortality.

Acknowledgements

This work was supported by European Union grant QLRT-2001-01034 (Novaflu). The authors thank T.M. Bestebroer and M.I.J. Spronken for excellent technical assistance.

Chapter

6

Assessment of the extent of variation in influenza A virus cytotoxic T-lymphocyte epitopes using virus-specific $CD8^+$ T-cell clones

E.G.M. Berkhoff • M.M. Geelhoed-Mieras • R.A.M. Fouchier
A.D.M.E. Osterhaus • G.F. Rimmelzwaan

Department of Virology and postgraduate school Molecular Medicine, Erasmus Medical Center, Rotterdam, The Netherlands

Journal of General Virology 2007; 88:530-5

The influenza A virus nucleoprotein (NP) and matrix protein are major targets for human virus-specific cytotoxic T-lymphocyte (CTL) responses. Most of the CTL epitopes that have been identified so far are conserved. However, recently sequence variation in CTL epitopes of the NP has been demonstrated associated with escape from virus-specific CTL. To assess the extent of variation in CTL epitopes during influenza A virus evolution, 304 CTL clones derived from six study subjects were obtained with specificity for an influenza A/H3N2 virus isolated in 1981. Subsequently, the frequency of the CTL clones was determined that failed to recognize a more recent influenza virus strain isolated in 2003. In four out of six study subjects CTL were found specific for variable epitopes, accounting for 2.6% of all CTL clones. For some of these CTL clones the minimal epitope and the residues responsible for abrogation of T-cell recognition were identified.

Cytotoxic T-lymphocytes (CTL) play an important role in the control of virus infections, including those caused by influenza viruses (185). Targets for CTL responses like the viral nucleoprotein (NP) and matrix protein are considered as candidate vaccines, because of their conserved nature. The use of conserved proteins could provide protective immunity against drift variants or viruses with novel subtypes (246, 283). Indeed, the majority of CTL epitopes that have been identified are conserved (185). However, the use of prototypic strains in these studies may have resulted in a bias towards the preferential identification of conserved CTL epitopes (61-63, 87, 125, 158, 160, 216, 245, 263). In addition, functional constraints of these proteins may limit variation of epitopes and thus limit escape from virus-specific CTL (17, 209, 210). We recently have shown that a number of influenza A virus epitopes are variable and that amino acid substitutions in these epitopes are associated with escape from virus-specific CTL. An amino acid substitution at position 384 of the nucleoprotein (R384G), which is at the anchor residues of the HLA-B*2705-restricted $NP_{383-391}$ and HLA-B*0801-restricted $NP_{380-388}$ epitopes, abrogated the recognition of the epitopes by specific CTL (16, 88, 211, 253). Other variable epitopes include the HLA-B*3501-restricted epitope $NP_{418-426}$ that displayed extensive amino acid variation at T-cell receptor contact residues (26) and the $NS1_{122-130}$ epitope (158) (Berkhoff, unpublished observation). Because examples of mutations in influenza A virus CTL epitopes are still anecdotal, we wished to determine the extent of variation in CTL epitopes during influenza A virus evolution.

In a previous study, we showed that relatively more non-synonymous mutations occurred in known influenza A virus CTL epitopes located in the NP than in the rest of this protein, which is suggestive for selective pressure on CTL epitopes (17, 137, 178, 184). In order to obtain an impression of the number of epitopes subject to amino acid variation that would affect their binding to their corresponding HLA-molecules, predicted epitopes in a historic strain of influenza A virus (H3N2) were compared with their sequence in a more recent strain. To this end, the PB2, PB1, PA and NP sequence of influenza virus A/Hong

Kong/1/68 (158) was used in the epitope prediction algorithms BIMAS and SYFPEITHI (at http://bimas.dcrt.nih.gov/molbio/hla_bind/ and www.syfpeithi.de, respectively). Epitopes were predicted for HLA-alleles for which no epitopes had been described previously (non-HLA-A*0101, -A*0201, -B*0701, -B*0801, -B*2705, -B*3501). Three hundred thirty nine epitopes were predicted with a SYFPEITHI score ≥ 20 and 840 epitopes were predicted with a BIMAS ranking ≤ 10. These predicted epitopes were compared with their amino acid sequences of more recent influenza (H3N2) strains A/Shiga/25/97 (PB2, PB1, PA) and A/Christchurch/39/2000 (NP). Seventy-seven of the predicted epitopes displayed amino acid variation at anchor residues, resulting in a considerable decrease in score and/or ranking of these epitopes. Ten predicted wild type epitopes were selected and synthesized as peptides, based on the availability of HLA-typed peripheral blood mononuclear cells (PBMC) and the emergence of the variant epitope after 1980. However, non of these ten wild type peptides were recognized by *in vitro* expanded polyclonal T-cell populations specific for influenza virus A/Hong Kong/1/68 as measured in IFN-γ ELISPOT assays (data not shown). Unfortunately, there is a poor correspondence between predicted and experimental binding of peptides to MHC class I-molecules, and it was reported that these programs could produce a considerable number of false positives (5). In addition, only a minority of predicted epitopes proved to be immunogenic (240). This approach would only allow the identification of amino acid substitutions at anchor residues. Since it is known that virus also can escape from CTL by mutations at T-cell receptor contact residues (26), we decided also to follow an empirical approach.

In order to determine the frequency of virus-specific CTL, which lost recognition of their epitope during influenza A virus evolution, PBMC of six HLA-typed study subjects between 35 and 55 years of age were stimulated *in vitro* with an influenza virus that was isolated in 1981 (A/Netherlands/4791/81 (H3N2), 1981-virus). This way, 1981-virus specific $CD8^+$ CTL were expanded for eight days in the presence of rIL-2 as described previously (25). We selected study subjects with HLA-haplotypes (table 1) for which no CTL epitopes had been described previously in order to prevent the identification of known conserved or variable epitopes. From the *in vitro* expanded PBMC a total of 304 virus-specific CTL clones were obtained by limiting dilution and subsequent non-specific stimulation with PHA (table 1). Cells were cultured at a density of 0.3, 1 and 3 cells per well in the presence of irradiated allogenic PBMC and selected Epstein-Barr virus transformed B-lymphoblastoid cells lines (BLCL) as described previously (253). Next, all 1981-virus specific $CD8^+$ CTL clones were tested for recognition of autologous BLCL infected with a virus that was isolated in 2003 (A/Netherlands/9/2003 (H3N2), 2003-virus). Both the 1981-virus and the 2003-virus were propagated in cell culture only, excluding amino acid variation based on difference in passage history of these viruses. In four out of the six study subjects, CTL clones were identified that were unable to recognize the 2003-virus infected cells (table 1). A total of eight out of the 304 (2.6%) 1981-virus specific CTL failed to recognize the recent

Table 1. Frequency of 1981-virus specific CTL that failed to recognize the 2003-virus.

Subject number	HLA-haplotype	Influenza virus specific clones/ clones tested	Clones recognizing a variable epitope
4108	A*0301,2402; B*3701,5601; C*0102,0602	34/180	0/34
0435	A*26HW,6801; B*3801,4002; C*0202,12CF	55/200	2/55
0187	A*0301,2301;B*1501,5002;C*0304,0602	40/165	1/40
6104	A*1101,3001;B*3801,5501;C*0303,12CF	36/180	0/36
6469	A*03AHY,2403;B*3801,7301;C*12CF,15XX	82/167	1/82
3909	A*0301,3101;B*1503,5101;C*12CF,15XX	57/178	4/57
Total		304/1070	8/304 (2.6%)

strain of influenza A virus. Repeated infections in these individuals might have decreased the frequency of CTL specific for the original version of variable epitopes relative to those specific for conserved epitopes that were boosted during repeated infections, which may result in an underestimation of the proportion of CTL that originally lost recognition. Thus, the number of examples of variable CTL epitopes in the influenza A virus nucleoprotein is increasing, which indicates that there is more variation in these CTL epitopes than was thought previously (185).

For some of the 1981-virus specific $CD8^+$ CTL clones long-term cultures could be established. For these clones the MHC class I-restriction and the minimal epitope could be identified. To this end, the CTL clones were stimulated with autologous BLCL that were transfected with plasmids expressing individual influenza virus genes to identify the target protein. Using epitope prediction algorithms and the recognition patterns with BLCL infected with influenza virus for which the amino acid sequence was available (table 2), the regions in which the probable epitopes were located were identified. With partially overlapping and truncated synthetic peptides the minimal epitopes were determined (figure 1A, D, G). For subject # 0435, two HLA-B*4002-restricted CTL clones were obtained specific for the $NP_{251-259}$ epitope AEIEDLIFS. Indeed, these clones ($NP_{251-259}$ /1981) failed to recognize the 2003-virus with the S259L mutation as well as the mutant peptide AEIEDLIFL (data is shown for one of these clones, figure 1A, B, C). The HLA-B*1503-restricted CTL clone specific for the $NP_{103-111}$ epitope KWMRELVLY ($NP_{103-111}$/1981), obtained from subject #3909 failed to recognize the K103R mutant peptide RWMRELVLY (figure 1G, H). The other three CTL clones obtained from this study subject could not be tested further because of poor yields in cell number upon re-stimulation.

It has been demonstrated that amino acid substitutions at T-cell receptor contact residues, but not at anchor residues, can lead to T-cell responses with specificity for the new variant of the epitope (26, 27). Therefore, we also stimulated PBMC of subject #0435 with the 2003-virus and generated $CD8^+$ CTL clones specific for the 2003-virus that were tested for their reactivity with the 1981-virus. Seven out of 76 influenza virus A/Nether-

Table 2. Reactivity of 1981-virus specific CTL clones with autologous BLCL infected with various influenza virus strains.

CTL clone	$NP_{246-265}$ Amino acid sequence	Influenza virus strain	Recognition[a]
$NP_{251-259}$/1981	RNPGNAEFEDLTFLARSALI	A/Puerto Rico/8/34	No
	-------I---I--------	A/Hong Kong/1/68	No
	--------------------	A/X-31	No
	-------I---I-S------	A/Netherlands/4791/81	Yes
	-------I---I-S------	A/Netherlands/178/95	Yes
	-------I---I--------	A/Netherlands/9/03	No
	-------I---I--------	A/Netherlands/20/03	No
	-------I---I--------	A/Netherlands/213/03	No

	NP_{98-116} Amino acid sequence	Influenza virus strain	Recognition[a]
$NP_{103-111}$/1981	RRVNGKWMRELILYDKEEI	A/Puerto Rico/8/34	Yes
	K--D-------V-------	A/Hong Kong/1/68	Yes
	--------------------	A/X-31	Yes
	K--D-------V-------	A/Netherlands/4791/81	Yes
	K--D-R-----V-------	A/Netherlands/178/95	No
	K--D-R-----V-------	A/Netherlands/9/03	No
	---D-------V-------	A/Netherlands/20/03	Yes
	---D-------V-------	A/Netherlands/213/03	Yes

a Recognition was determined by ELISPOT assays.

lands/9/2003-specific CTL clones failed to recognize the 1981-virus. Of special interest, one of these 2003-virus specific CTL clones was specific for the HLA-B*4002-restricted $NP_{251-259}$ variant epitope, which was not recognized by $NP_{251-259}$-specific CTL clones directed to the 1981-virus (see above). This CTL clone cross-reacted with the 1981-variant of the peptide with 30-fold lower functional avidity than the homologous 2003-version of the peptide (figure 1D, E, F). Most likely, the 1981-peptide concentration required for the activation of these 2003-virus CTL is higher than achieved during infection of target cells with the 1981-virus, which explains the observation that 1981-virus infected cells are not recognized by this 2003-virus specific CTL clone.

Thus, a mutant version of the epitope emerged that escaped from CTL specific for the 1981-variant of the epitope and that induced a CTL response that partially cross-reacted with the 1981-variant of the epitope. A similar finding was observed previously for the HLA-B*3501-restricted $NP_{418-426}$ epitope that displays extensive variation at T-cell receptor contact residues (27). The other six 2003-virus specific CTL clones were all directed to the HLA-A*6801-restricted $NP_{145-152}$ epitope (DATYQRTR) which contained the T146A substitution. The full meaning of variation in this CTL epitope is unclear at present, since CTL clones specific for the 1981-variant of the epitope were not available.

As shown in figure 1I, the amino acid substitutions in the $NP_{251-259}$ epitope reached fixation rapidly during influenza A virus evolution and the 259L variant has been circulating since 1997. Variants of the $NP_{103-111}$ epitope replaced each other more frequently; from 1968

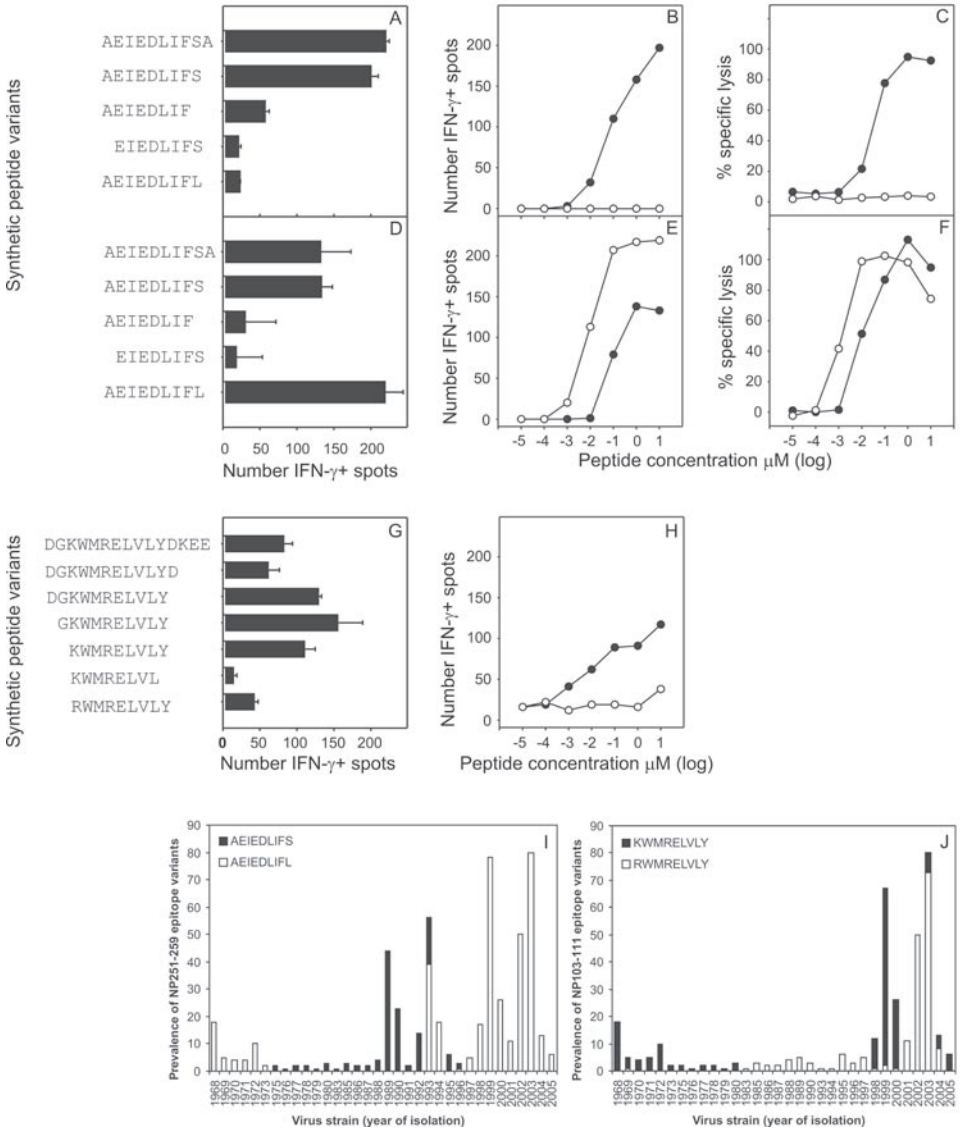


Figure 1. Recognition of wild type and mutant versions of epitopes $NP_{251-259}$ and $NP_{103-111}$ by virus-specific CTL. The minimal epitopes that were recognized by CTL clones specific for the $NP_{251-259}$ 1981-wild type epitope (A), the $NP_{251-259}$ 2003-variant epitope (D), and the 1981-wild type $NP_{103-111}$ epitope (G) were determined with partially overlapping and truncated peptides as indicated. Next, the functional avidity of CTL clones specific for the $NP_{251-259}$ wild type epitope (B and C), the $NP_{251-259}$ variant epitope (E and F) and the wild type $NP_{103-111}$ epitope (H) was assessed for the wild type epitope derived from the 1981-virus (●) and the mutant version of the epitope derived from the 2003-virus (○) by ELISPOT assays (B, E, H) or chromium-release assays (C and F). Autologous BLCL pulsed with serial dilutions of the respective peptides were used as stimulator cells for use in the ELISPOT assays (10^3 CTL clone stimulated with 2.5×10^4 stimulator cells) or as target cells in chromium-release assays (effector-to-target ratio of 10). The percentage specific lysis was calculated with the following formula: ((experimental release-spontaneous release) / (maximum release-spontaneous release)) × 100%. The prevalence of the $NP_{251-259}$ and $NP_{103-111}$ epitope variants during influenza A (H3N2) virus evolution is shown in figures I and J. Indicated are the total numbers of $NP_{251-259}$ (I) and $NP_{103-111}$ (J) variant sequences per year available in the Influenza Sequence Database (www.flu.lanl.gov) (158). Black bars indicate the epitope amino acid sequence as in the 1981-virus and the white bars indicate the epitope amino acid sequence as in the 2003-virus.

until 1983 and from 1998 until 2001 the KWMRELVLY variant was most predominant, whereas the RWMRELVLY variant was found predominantly from 1983 until 1998 and from 2001 until 2004, and was subsequently replaced by the original variant of the epitope (figure 1J). The prevalence of the mutant viruses did not correlate with the phylogenetically distinct lineages of co-circulating influenza A viruses (116). The rapid fixation of mutations in influenza A virus CTL epitopes was explained previously by small selective advantages and population dynamics in a theoretical model (88). The alternating fixation of wild type and mutant variants of the $NP_{251-259}$ and the $NP_{103-111}$ epitopes in time is of interest and may reflect a dynamic interplay between immune evasion and intrinsic viral fitness. It is possible that the relatively low prevalence of HLA-B*1503 and -B*4002 in the human population (0.87 and 2.94% on average in the Caucasian population) (163) may have contributed to incomplete immune pressure on these viruses, allowing the wild type variant to re-emerge repeatedly.

The antigenic changes in the NP responsible for escape from virus-specific CTL may contribute to the successful persistence of influenza A viruses in the human population. Furthermore, these changes should be taken into account in the development of vaccines, which aim at the induction of protective T-cell responses.

Acknowledgements

This work was supported by European Union grant QLRT-2001-01034 (Novaflu). We thank R. van Beek for technical assistance. We also acknowledge B. Tomson for the selection of HLA-typed blood donors (Sanquin Bloodbank).

Chapter

7

The loss of immunodominant epitopes affects IFN-γ production and lytic activity of the human influenza virus-specific cytotoxic T-lymphocyte response *in vitro*

E.G.M. Berkhoff • M.M. Geelhoed-Mieras • E.J. Verschuren
C.A. van Baalen • R.A. Gruters • R.A.M. Fouchier
A.D.M.E. Osterhaus • G.F. Rimmelzwaan

Department of Virology and postgraduate school Molecular Medicine,
Erasmus Medical Center, Rotterdam, The Netherlands

Clinical and Experimental Immunology 2007 (in press)

Influenza A viruses can exploit a variety of strategies to evade host immunity, including the acquisition of mutations in cytotoxic T-lymphocyte (CTL) epitopes. The loss of CTL epitopes may impair the functionality of the CTL response and could result in reduced cytokine production and lytic activity against virus-infected cells. In the present study, we examined the effect of the loss of the HLA-B*3501-restricted $NP_{418-426}$ epitope on IFN-γ-production and lytic activity of the human CTL response *in vitro*. The $NP_{418-426}$ epitope is highly variable and extensive amino acid variation at T-cell receptor contact residues has led to repeated evasion from $NP_{418-426}$-specific CTL. We used reverse genetics to generate recombinant influenza viruses with variants of the $NP_{418-426}$ epitope. These viruses were used to stimulate peripheral blood mononuclear cells obtained from six HLA-B*3501-positive study subjects in order to expand virus-specific CTL. Loss of the $NP_{418-426}$ epitope resulted in a significant reduction of IFN-γ-expressing $CD8^+$ T-cells upon re-stimulation with virus-infected MHC class I-matched cells, similar to that observed previously after the loss of the HLA-B*2705-restricted $NP_{383-391}$ epitope. In addition, the effect of the loss of the $NP_{418-426}$ epitope on the lytic activity of the virus-specific CTL response was assessed. Also this functional property of the virus-specific CTL response was significantly affected by the loss of this and the $NP_{383-391}$ epitope, as was determined using the newly developed Fluorescent-Antigen-Transfected-Target cell-Cytotoxic T-Lymphocyte (FATT-CTL) assay. These findings indicate that the loss of single immunodominant epitopes affects the functionality of the virus-specific CTL response significantly.

Introduction

Cytotoxic T-lymphocytes (CTL) play an important role in the control of viral infections, including those caused by influenza viruses. In mice, mortality caused by influenza virus infection was reduced and more efficient viral clearance was shown upon adoptive transfer of virus-specific CTL (159, 237, 267, 276, 277). In addition, mice lacking MHC class I-restricted $CD8^+$ T-cells show delayed viral clearance and increased mortality (12, 222, 266). In humans, the level of influenza virus-specific CTL activity correlated with the rate of viral clearance upon infection (168, 169). However, viruses can escape from recognition by specific CTL by accumulation of amino acid substitutions at sites important for epitope processing, MHC class I-binding, and/or T-cell receptor (TCR) interaction (138, 183, 197). Also influenza A viruses can evade CTL responses by amino acid substitutions in CTL epitopes (26, 185, 202, 211, 253). The R384G substitution in the influenza virus nucleoprotein (NP) is at the anchor residue of the HLA-B*0801-restricted $NP_{380-388}$ epitope and the HLA-B*2705-restricted $NP_{383-391}$ epitope, and prevented binding of the epitopes to their corresponding MHC class I-molecules and recognition by specific CTL (16, 211, 253). Also for influenza virus, amino acid variation at TCR contact residues can result in evasion from specific CTL.

Examples of this are amino acid substitutions at positions 421, 422, 423, and 425 of the hypervariable HLA-B*3501-restricted $NP_{418-426}$ epitope. Influenza A viruses with variation in the $NP_{418-426}$ epitope emerged over time, and $NP_{418-426}$-specific CTL directed against earlier virus strains failed to recognize more recent variants of influenza A virus, indicating escape from CTL immunity (26). The high degree of amino acid variability is probably driven by CTL that recognize their epitope with high functional avidity (24). Amino acid substitutions at anchor residues were not observed in the $NP_{418-426}$ epitope, which may be explained by functional constraints. Indeed, we recently showed that the replacement of the anchor residue at position 419 of the NP impaired viral fitness (17). For some $NP_{418-426}$ epitope variants, a small proportion of the CTL directed against one epitope variant cross-reacted with another epitope variant (27, 101). In a subsequent infection with an influenza virus with the corresponding variant epitope these cross-reactive CTL may be further expanded, indicating that immune evasion may only be temporary.

In the present study, we examined the impact of the loss of the $NP_{418-426}$ epitope on the human influenza virus-specific CTL response *in vitro*. To this end, influenza viruses with various $NP_{418-426}$ epitope sequences were generated by reverse genetics. Since the next evading amino acid substitution within the $NP_{418-426}$ epitope cannot be predicted, we used the historical epitope sequence of influenza virus A/PR/8/34 (LPFDRTTIM, further referred to as DRI), isolated in 1934, and the sequence of an epitope variant that circulated from 1957 till 1972 (LPFDKPTIM, further referred to as DPI) for the generation of viruses "without" the $NP_{418-426}$ epitope. Both epitope sequences are not recognized by CTL specific for the current epitope variant (LPFEKSTVM, further referred to as ESV) (26, 27). In addition, we hypothesized that these natural sequences would not be detrimental to viral fitness and would yield viable viruses. We have shown previously that the loss of the HLA-B*2705-restricted $NP_{383-391}$ epitope affected the human influenza virus-specific CTL response *in vitro* significantly, as measured by the number of IFN-γ producing $CD8^+$ T-lymphocytes (16). However, for the elimination of virus infected cells and viral clearance, the lytic activity of virus-specific CTL may be more important (99, 179, 180, 248). Therefore, we investigated the effect of the loss of the HLA-B*2705-restricted $NP_{383-391}$ and the HLA-B*3501-restricted $NP_{418-426}$ epitope on the lytic activity of the influenza virus NP-specific response, in addition to the effect on IFN-γ production. Using *in vitro* stimulated peripheral blood mononuclear cell (PBMC) cultures, we assessed the virus-specific $CD8^+$ T-cell response directed against influenza virus with or without the $NP_{418-426}$ epitope by tetramer and intracellular IFN-γ staining, and flow cytometry. For the measurement of the lytic activity of clonal and *in vitro* expanded polyclonal T-cell populations, we used the newly developed Fluorescent-Antigen-Transfected-Target cell-Cytotoxic T-Lymphocyte (FATT-CTL) assay, which is based on the use of target cells transfected with plasmids expressing viral proteins fused to green fluorescent protein (GFP) (249). This procedure allowed the detection of a significant reduction of the NP-specific lytic activity of the human influenza virus-specific CTL response caused

by the loss of the HLA-B*2705-restricted $NP_{383-391}$ and the HLA-B*3501-restricted $NP_{418-426}$ epitopes.

Material and methods

Plasmids

For the generation of recombinant influenza viruses, a bidirectional reverse genetics system based on influenza virus A/Netherlands/178/95 (A/NL/178/95; H3N2) as previously described (17), was used. Site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA) of the NP segment was performed to alter the $NP_{383-391}$ or $NP_{418-426}$ sequence, as listed in table 1. For the construction of FATT-CTL assay vectors, the variant NP genes were cloned into the multiple cloning site of Living Colors vector pEGFP-N1 (Becton Dickinson, Alphen a/d Rijn, The Netherlands) in frame with the open-reading frame of GFP, using restriction enzymes NheI en AgeI. Read-through of the GFP gene was achieved by deleting the stopcodon of the NP gene. Sequence analysis was performed for all recombinant plasmids, using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), according to the instructions of the manufacturer. All PCR primer sequences and plasmid maps are available on request.

Generation of viruses

The recombinant bidirectional plasmids were transfected into 293T cells, using the calcium phosphate precipitation method as described previously (58). After 48 hours, culture supernatants were harvested, and used for subsequent infection of confluent Madin-Darby Canine Kidney (MDCK) cells (16). After three days, culture supernatants were harvested, cleared by low speed centrifugation, aliquoted and stored at -80 °C until use. The recombinant viruses were designated influenza virus A/NL/95-ESV, -DPI and -DRI corresponding with the substitutions as indicated in table 1. In order to confirm the introduction of the mutations and to exclude the introduction of second site mutations, the nucleotide sequences of the corresponding full-length genes were assessed. Infectious virus titers were determined as previously described (208) (table 1). The generation of the recombinant influenza viruses with or without the $NP_{383-391}$ epitope, designated influenza virus A/NL/94-384R and A/NL/94-384G respectively, was described previously (16).

Peripheral Blood Mononuclear Cells (PBMC)

Genetic subtyped PBMC from healthy blood donors, between 35 and 50 years of age, were isolated from heparinized blood (Sanquin Bloodbank, Rotterdam, The Netherlands) by density gradient centrifugation using Lymphoprep (Axis-Shield PoC, Oslo, Norway) and were cryopreserved at -135 ºC. All study subjects had serum antibodies against one or more influ-

Table 1. Substitutions introduced in influenza A virus CTL epitopes.

Epitope	Amino acid substitutions									Designated name	Year of isolation	$TCID_{50}$/ml[a]
$NP_{383-391}$	S	R	Y	W	A	I	R	T	R	384R	<1993	6.875
		G								384G	1993-2006	7.000
$NP_{418-426}$	L	P	F	E	K	S	T	V	M	ESV	1980-2004	7.175
				D		P		I		DPI	1957-1972	7.000
				D	R	T		I		DRI	1934	7.125

a Upon rescue of recombinant viruses containing the respective epitope sequence, the infectious virus titers were determined and expressed as $TCID_{50}$/ml.

enza A virus strains (H1N1 or H3N2) (25, 27), indicating one or more exposures to influenza A virus. Permission to use the PBMC for scientific research was obtained from the blood donors by informed consent.

In vitro stimulation of PBMC with influenza A viruses

PBMC were resuspended in RPMI 1640 medium (Cambrex, East Rutherford, NJ, USA) supplemented with 10 % Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (antibiotics) (R10F). Five million PBMC were infected with recombinant influenza virus at a multiplicity of infection (MOI) of three in a volume of 5 ml R10F as described previously (25). After one hour at 37 ºC, the cells were resuspended in RPMI 1640 medium supplemented with 10 % pooled human serum (Sanquin Bloodbank), antibiotics and 20 µM β-mercaptoethanol (R10H) and added to non-infected PBMC at a ratio of 1:1 in a 25 cm^2 culture flask. After 48 hours of stimulation rIL-2 was added to a final concentration of 50 U/ml. Subsequently, the cells were cultured for an additional 6-8 days.

Isolation of $CD8^+$ cells

$CD8^+$ cells were isolated from the *in vitro* expanded PBMC cultures by magnetic sorting, using a CD8 MicroBeads kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the instructions of the manufacturer. The isolated $CD8^+$ cells were used as effector cells in the FATT-CTL assays.

$CD8^+$ T-cell clones

Generation of $CD8^+$ T-cell clones directed against the HLA-A*0101-restricted epitope NP_{44-52}, the HLA-A*0201-restricted epitope $M1_{58-66}$, the HLA-B*2705-restricted epitope NP_{383-}

$_{391}$, and the different variants of the HLA-B*3501-restricted epitope $NP_{418-426}$ was described previously (27, 253).

Target cells

B-lymphoblastoid cell lines (BLCL), established as described previously (212), and an HLA-B*3501-transfected C1R cell line kindly provided by Dr. M. Takiguchi, were used as target cells. For infection with the recombinant influenza viruses, 10^6 target cells were infected at a MOI of three in a volume of 1 ml. After incubation for one hour at 37 ºC the cells were resuspended in R10F and incubated for 16-18 hrs. For exogenous protein labeling 50 μg recombinant influenza virus nucleoprotein (rNP), derived from influenza virus A/PR/8/34 (rNP-PR), A/HK/2/68 (rNP-HK) or A/NL/18/94 (rNP-NL), was added to 10^6 cells in 1 ml R10F, as described previously (254). Peptide labeling was performed by incubating 10^6 cells/ml overnight with 5 μM peptide in R10F. Peptides were manufactured, HPLC-purified (immunograde, 85% purity) and analyzed with mass spectrometry (Eurogentec, Seraing, Belgium). For transfection, 8 μg plasmid DNA (1 μg/μl) was added to 2×10^6 BLCL, resuspended in 100 μl solution V (Amaxa Biosystems, Cologne, Germany) at room temperature. Subsequently, the cells were electroporated in 2 mm electroporation cuvettes (Amaxa Biosystems), using program T16 in the Nucleofector™ (Amaxa Biosystems). Immediately after electroporation, 500 μl R10F (37ºC) was added. Cells were transferred to a 24-wellsplate for overnight incubation.

Tetramer staining and flow cytometry

Tetramer staining was performed on the *in vitro* influenza virus expanded PBMC cultures, as described previously (27). The allophycocyanin (APC)-conjugated tetramers consisted of HLA-B*3501 molecules complexed with the $NP_{418-426}$ epitope variants ESV and DPI. In addition, phycoerythrin (PE)-conjugated tetramers were used, consisting of HLA-A*0101 and -A*0201 molecules complexed with the NP_{44-52} or $M1_{58-66}$ epitope respectively. Two hundred thousand cells were stained with tetramer for 20 minutes at room temperature, followed by a 10-minute incubation with monoclonal antibody (MAb) directed to CD3 (Becton Dickinson) and CD8 (Dako, Glostrup, Denmark). At least 10^4 $CD8^+$ events were acquired using a FACSCalibur (Becton Dickinson) flow cytometer. The data were analyzed using the software program Cell Quest Pro (Becton Dickinson).

Intracellular IFN-γ staining and flow cytometry

The *in vitro* influenza virus expanded PBMC cultures were adjusted to a concentration of 10^6 cells/ml in R10F supplemented with Golgistop (monensin; Pharmingen, Alphen a/d Rijn, The Netherlands). Hundred thousand effector cells were incubated with 2×10^5 stimulator cells, which were infected with influenza virus at a MOI of three, labeled with 50 μg

influenza virus rNP, pulsed with peptides at a concentration of 5μM or left untreated, for 6 hours at 37 ºC in U-bottom plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands). Subsequently intracellular IFN-γ-staining was performed as described previously (16). In brief, the cells were washed with PBS containing 2% FCS (P2F) and Golgistop, stained with MAb directed to CD3 and CD8, fixed and permeabilized with cytofix and cytoperm (Pharmingen) and stained with a MAb specific for IFN-γ (Pharmingen). Approximately 5×10^3 $CD3^+$ $CD8^+$ events were acquired using a FACSCalibur flow cytometer. The data were analyzed using the software program Cell Quest Pro.

Chromium-release assay

Chromium-release assays were performed as described previously (25). In brief, 7.5×10^5 cells per target were labeled with 75 μCi $Na_2[^{51}Cr]O_4$, and incubated with $CD8^+$ T-cell clone at effector-to-target (E:T) ratios of 10, 5, 2.5, and 1. Target cells were also incubated with 10% Triton X-100 or R10F to determine the maximum and spontaneous release, respectively. After 4 hrs incubation, the supernatants were harvested (Skatron instruments, Sterling, VA, USA) and radioactivity was measured by gamma-counting. The percentage specific lysis was calculated with the following formula: ((experimental release-spontaneous release) / (maximum release-spontaneous release)) × 100%. The chromium-release assays were performed in quadruplicate and the data were presented as the average.

Fluorescent-Antigen-Transfected Target cell-Cytotoxic T-Lymphocyte (FATT-CTL) assay

FATT-CTL assays were performed as described previously (249). In brief, 1000 viable GFP-positive target cells were incubated with CTL clone at E:T ratios of 10, 3, 1, 0.3 and 0.1 for 4hrs at 37ºC, or with $CD8^+$ cells isolated from *in vitro* expanded PBMC cultures at E:T ratios of 10, 3, 1 and 0.3 for 4hrs at 37ºC, or with *ex vivo* non-stimulated PBMC at E:T ratios of 100, 30, 10 and 3 for 18hrs at 37ºC in 200μl R10F supplemented with rIL-2 (50U/ml). The flow rate (μl/s) was determined by calibrated number of beads (TruCOUNT tubes; Becton Dickinson). EDTA (final concentration 2.5mmol/L) and TOPRO-3 iodide (final concentration 25nmol/L; Molecular Probes, Invitrogen, Paisley, UK) were added to prevent clustering of cells and to identify viable (TOPRO-3 negative) cells respectively, and the samples were incubated for an additional 20 minutes at 37ºC. Each sample was acquired for a fixed period of 60 seconds, using a FACSCalibur flow cytometer. The forward scatter acquisition threshold was set to exclude debris. The FL-1 (GFP) acquisition threshold was set to reduce GFP-negative events. A constant flow rate was monitored in a time-event histogram. The data were analyzed using the software program Cell Quest Pro. The percentage specific lysis was calculated with the following formula: ((viable GFP^+ events without effector- viable

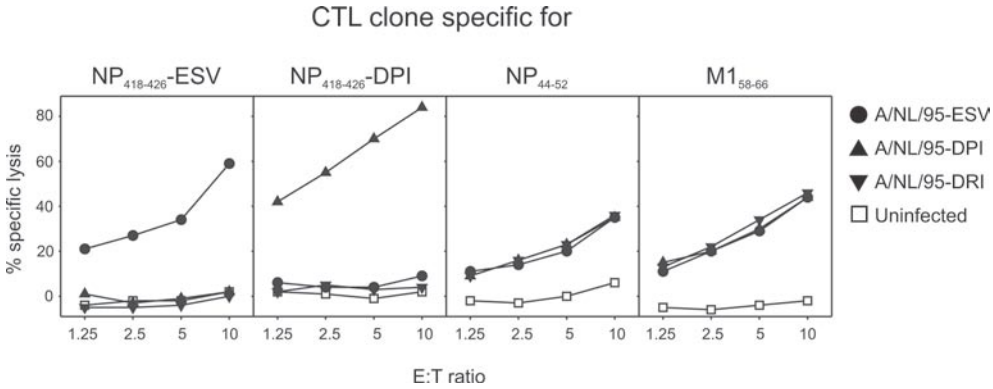


Figure 1. Recognition of BLCL infected with recombinant influenza viruses by CTL clones. HLA-A*0101, -A*0201 and -B*3501-positive BLCL were infected with influenza virus A/NL/95-ESV (●), -DPI (▲) or -DRI (▼), or left untreated (□), and used as target cells for $CD8^+$ T-cell clones specific for the HLA-A*0101-restricted NP_{44-52} epitope, the HLA-A*0201-restricted $M1_{58-66}$ epitope or the HLA-B*3501-restricted $NP_{418-426}$ epitopes ESV or DPI in a classical chromium-release assay. CTL clones were added at different E:T ratios as indicated, and specific lysis was calculated. The chromium-release assay was performed in quadruplicate and the data are presented as the average.

GFP^+ events with effector) / (viable GFP^+ events without effector)) × 100%. The FATT-CTL assays were performed *in duplo* and the data were presented as the average.

Results

Replication and recognition of influenza $NP_{418-426}$ variant viruses

By reverse genetics, identical influenza viruses A/NL/178/95 were generated which differed only regarding their $NP_{418-426}$ epitope sequence. Mutations were introduced based on historical epitope sequences, since it was anticipated that these sequences would yield viable viruses. Indeed A/NL/178/95 viruses with the $NP_{418-426}$ epitope sequence of influenza virus A/PR/8/34, LPFDRTTIM, could be rescued as were viruses with the LPFDKPTIM variant that circulated from 1957 till 1972 and the homologous contemporary variant LPFEKSTVM. The recombinant viruses were designated influenza virus A/NL/95-ESV, -DPI and -DRI corresponding to the substitutions as indicated in table 1. All three viruses replicated to similar titers in MDCK cells and the growth kinetics of these viruses was similar (data not shown). Also the infection rate of BLCL was comparable, as determined by positive staining for NP (data not shown). BLCL that were infected with the three $NP_{418-426}$ variant viruses were all recognized equally well by $CD8^+$ T-cell clones specific for the conserved HLA-A*0101-restricted NP_{44-52} and HLA-A*0201-restricted $M1_{58-66}$ epitopes (figure 1), indicating that the mutations did not affect infection, antigen processing or presentation of the mutant viruses. In contrast, T-cell clones directed against the $NP_{418-426}$ epitope variants DPI and ESV only lysed cells that were infected with the influenza virus that contained the homologous epitope. Influenza virus A/NL/95-DRI infected cells were not recognized by either of these $NP_{418-426}$ epitope-specific CTL clones. Next, the three variant viruses were used

Table 2. HLA-A and -B genotypes of the six individuals included in this study, and proportions of virus- and $NP_{418-426}$ epitope-specific $CD8^+$ T cells in PBMC stimulated with influenza virus A/NL/95-ESV[a].

Symbols	Donor	HLA-A and -B haplotype	% of $CD8^+$ T cells specific for:		Relative proportion (%) specific for $NP_{418-426}$ ESV
			A/NL/95-ESV	$NP_{418-426}$ ESV	
●	3180	A*0101 A*0201 B*0801 B*3501	40.02	14.20	35.5
▲	6358	A*0101 A*0201 B*0801 B*3501	38.41	5.89	15.3
▼	0775	A*0101 A*0201 B*0801 B*3501	27.70	4.12	14.9
○	5507	A*0101 A*0301 B*0801 B*3501	14.75	1.50	10.2
△	5526	A*0101 A*0301 B*0801 B*3501	19.05	4.37	22.9
▽	6877	A*0101 A*0301 B*0801 B*3501	18.72	3.73	19.9

a Determined by IFN-γ expression in *in vitro* influenza virus A/NL/95-ESV stimulated PBMC after re-stimulation with A/NL/95-ESV-infected or $NP_{418-426}$ peptide-pulsed MHC class I-matched BLCL.

to stimulate PBMC obtained from HLA-B*3501-positive study subjects (table 2) in order to expand virus-specific $CD8^+$ T-lymphocytes. The proportion of ESV- and DPI-specific CTL within the $CD8^+$ population was determined by tetramer staining. A representative result obtained with the PBMC of an HLA-B*3501-positive study subject is shown in figure 2. On average 7.2% (ranging from 0.1 to 22.4%) of all $CD8^+$ T-cells were ESV-specific in PBMC cultures stimulated with influenza virus A/NL/95-ESV. In contrast, ESV-specific $CD8^+$ T-cells were virtually absent in PBMC cultures stimulated with influenza virus A/NL/95-DPI and -DRI. In the A/NL/95-ESV-stimulated PBMC cultures of two HLA-B*3501-positive study subjects (5507 and 5526), ESV-specific $CD8^+$ T-cells were undetectable, however, in the PBMC expanded after stimulation with influenza virus A/NL/95-DPI of one of these two study subjects (5526), 7.9% DPI-specific $CD8^+$ T-cells were detected. Tetramer staining for NP_{44-52} and $M1_{58-66}$ specific $CD8^+$ T-lymphocytes indicated that the infection of PBMC and subsequent stimulation was successful after stimulation with the three viruses, since the percentage of NP_{44-52} and $M1_{58-66}$ specific CTL was comparable in the three PBMC cultures of the individual study subjects. The results obtained for each individual study subject are shown in figure 3 (upper panels).

Effect of mutations in the $NP_{418-426}$ epitope on the virus-specific CTL response *in vitro*

To assess the effect of the loss of the $NP_{418-426}$ (ESV) epitope on the influenza virus-specific CTL response *in vitro*, PBMC obtained from the six HLA-B*3501-positive study subjects were stimulated with influenza viruses A/NL/95-ESV, -DPI or -DRI. After expansion, the number of virus-specific $CD8^+$ T-cells was determined upon induction of IFN-γ expression by re-stimulation with MHC class I-matched BLCL or HLA-B*3501-transfected C1R-cells

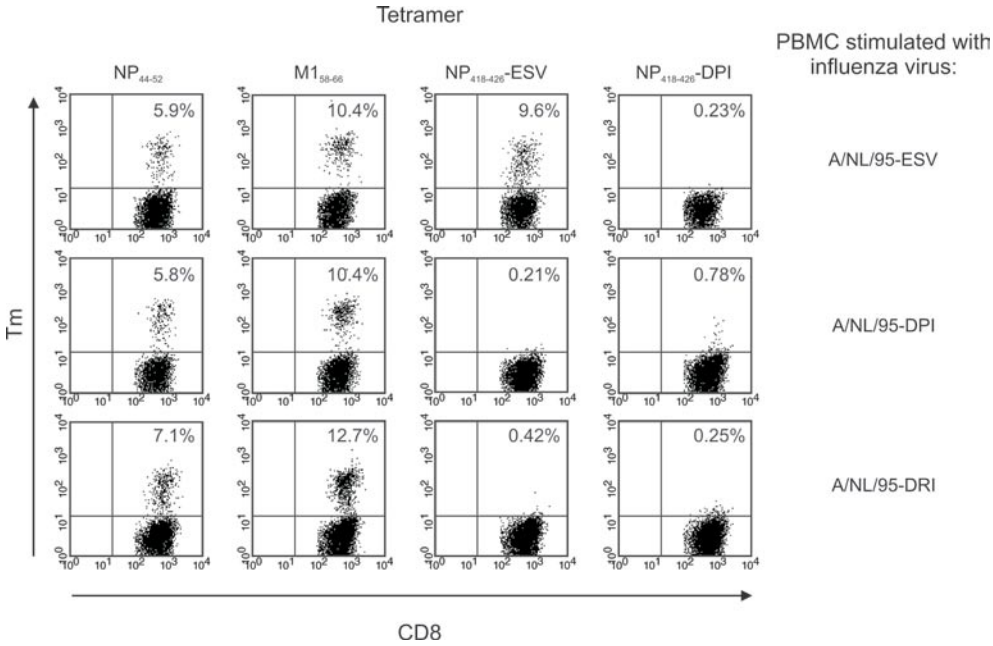


Figure 2. Tetramer staining of PBMC expansion cultures stimulated with various influenza virus variants. PBMC stimulated with influenza virus A/NL/95-ESV, -DPI or -DRI were stained with PE-conjugated tetramers consisting of HLA-A*0101 and -A*0201 molecules complexed with the NP_{44-52} or $M1_{58-66}$ epitope respectively, and APC-conjugated tetramers consisting of HLA-B*3501 molecules complexed with the $NP_{418-426}$ epitope variants ESV and DPI as indicated. The data of a representative experiment is shown (study subject 6358). The percentages indicate the proportion of tetramer-specific CTL within the $CD8^+$ T cell population.

that were infected with the three viruses. The percentage IFN-γ^+ $CD8^+$ T-cells after re-stimulation of the expanded PBMC cultures with peptide labeled stimulator cells correlated with the results of the tetramer staining (table 2 and figure 3 lower panels). In four out of the six study subjects similar T-cell recognition patterns were observed as shown in figure 4. The highest frequency of virus-specific CTL was observed in PBMC stimulated with influenza virus A/NL/95-ESV and re-stimulated with the same virus. Re-stimulation with influenza virus A/NL/95-DPI or -DRI resulted in IFN-γ expression in a smaller number of $CD8^+$ T-cells. Also after primary stimulation of PBMC with influenza virus A/NL/95-DPI or -DRI, the number of virus-specific CTL was reduced, regardless the influenza virus that was used for re-stimulation. For two study subjects (5507 and 5526) this was not the case, which correlated with the absence of $NP_{418-426}$ (ESV)-specific $CD8^+$ T-cells (figure 3 upper left panel). The difference in the percentage of IFN-γ^+ $CD8^+$ T-cells after re-stimulation with virus with the epitope or without the epitope (i.e. DRI) is plotted in figure 5, and ranged from no difference in the two subjects without a $NP_{418-426}$ ESV-specific response, to 12%. The average difference of 5.4% was statistical significant (ANOVA; $p<0.05$). In the influenza virus A/NL/95-DPI stimulated PBMC of the six HLA-B*3501-positive study subjects, no differences in the number of virus-specific cells was observed with influenza virus

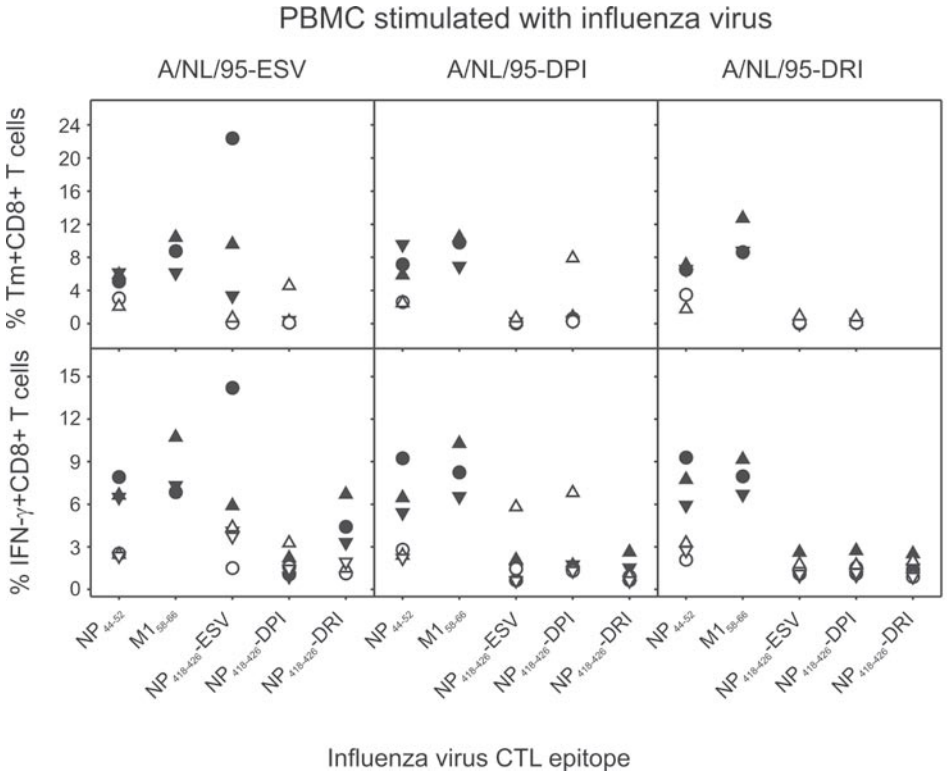


Figure 3. Recognition of $NP_{418-426}$ epitope variants by HLA-B*3501-positive PBMC cultures. PBMC obtained from HLA-B*3501-positive study subjects were stimulated with influenza virus A/NL/95-ESV, -DPI, and -DRI as indicated. The symbols correspond to individual study subjects listed in table 2. The cells were stained with tetramer (upper panels) as indicated. In addition, epitope-specific $CD8^+$ T cells were detected by staining for IFN-γ expression after re-stimulation with MHC class I-matched BLCL incubated with the peptides $M1_{58-66}$ (GILGFVFTL), NP_{44-52} (CTELKLSDY), or $NP_{418-426}$ (ESV, DPI, DRI) (lower panels). The $M1_{58-66}$-specific responses were only indicated for the subjects that were also HLA-A*0201-positive.

A/NL/95-DRI stimulated PBMC, apart from the PBMC obtained from study subject 5526 (figure 5B and D), who had an appreciable response specific for the $NP_{418-426}$ DPI epitope (figure 3). In the four HLA-B*3501-positive study subjects with a response to $NP_{418-426}$ ESV, the response to this epitope was immunodominant within the NP-specific response as was shown by using recombinant NP (figure 4 upper panels). It was also an immunodominant HLA-B*3501-restricted response as was shown by using virus-infected C1R-cells that only express HLA-B*3501 for re-stimulation (figure 4 lower panels). The loss of the $NP_{418-426}$ ESV epitope resulted in a relative reduction in virus-specific CTL response *in vitro* of 20-32% (figure 5C).

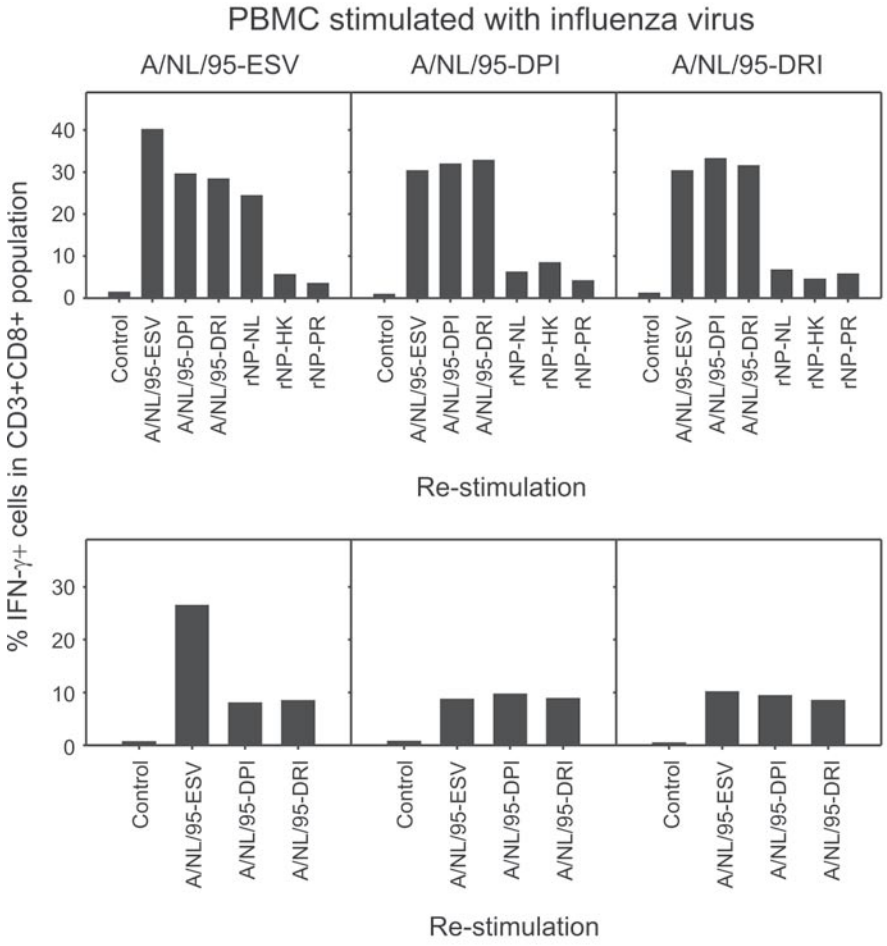


Figure 4. Percentages of virus-specific $CD8^+$ T cells in *in vitro* stimulated PBMC cultures. Percentages IFN-γ^+ $CD8^+$ T cells were determined in influenza virus A/NL/95-ESV, -DPI and -DRI stimulated PBMC cultures. The expanded PBMC were re-stimulated with MHC class I-matched BLCL (upper panels) or HLA-B*3501-transfected C1R-cells (lower panels) that were infected with influenza virus A/NL/95-ESV, -DPI or -DRI, or that were incubated with recombinant influenza virus NP (rNP), derived from influenza virus A/PR/8/34 (rNP-PR), A/HK/2/68 (rNP-HK) or A/NL/18/94 (rNP-NL). Virus-specific CTL were visualized after staining with monoclonal antibodies specific for CD3, CD8, and IFN-γ. The data of a representative experiment is shown (study subject 3180).

Establishment of the FATT-CTL assay for the detection of lytic activity of CTL

Since the lytic activity of CTL is probably most relevant for the elimination of infected cells and clearance of infections, we wished to investigate the effect of epitope loss on the lytic activity of the CTL response *in vitro*. First, the newly developed FATT-CTL assay was evaluated for this purpose. This assay allows the detection of target cell elimination by flow

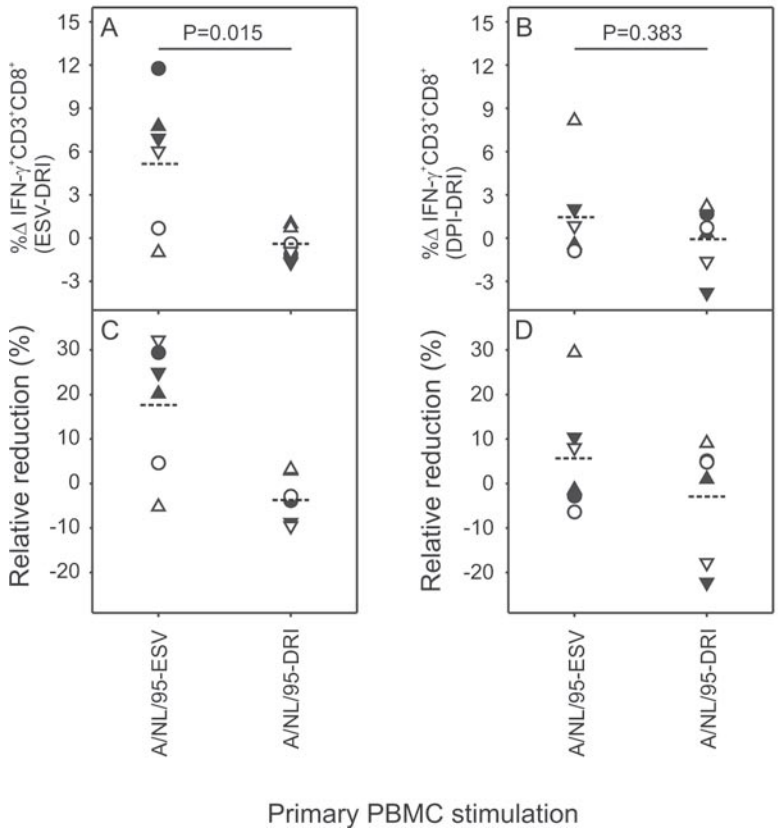


Figure 5. Reduction of the influenza virus-specific CTL response *in vitro* caused by amino acid variation within the $NP_{418-426}$ epitope. Reduction in the percentages of virus-specific IFN-γ^+ $CD8^+$ T cells in the recombinant virus stimulated PBMC cultures is shown for all six study subjects. Reduction was calculated by subtracting the percentage of IFN-γ^+ $CD8^+$ T cells after re-stimulation with influenza virus A/NL/95-DRI from the percentage of IFN-γ^+ $CD8^+$ T cells after re-stimulation with influenza virus A/NL/95-ESV (A, C) or -DPI (B, D). The percentage of IFN-γ^+ $CD8^+$ T cells is based on the analysis as depicted in figure 4 (upper panels). The relative reduction in the percentages of virus-specific IFN-γ^+ $CD8^+$ T cells in PBMC cultures (C, D) was calculated as follows: 100 × [(percentage of IFN-γ^+ $CD8^+$ T cells after re-stimulation with influenza virus A/NL/95-DRI × 100)/percentage of IFN-γ^+ $CD8^+$ T cells after re-stimulation with influenza virus A/NL/95-ESV or -DPI]. In all four panels, the average is shown (---). The symbols refer to the study subjects listed in table 2.

cytometry upon transfection of the target cells with plasmids expressing the gene of interest as GFP-fusion proteins. In figure 6, this is shown for a CTL clone specific for the $NP_{418-426}$ ESV epitope, and HLA-B*3501-positive BLCL cells transfected with control plasmid, only expressing GFP (upper panels), or plasmid expressing GFP-NP ESV (lower panels). From this data the extent of specific lysis could be calculated.

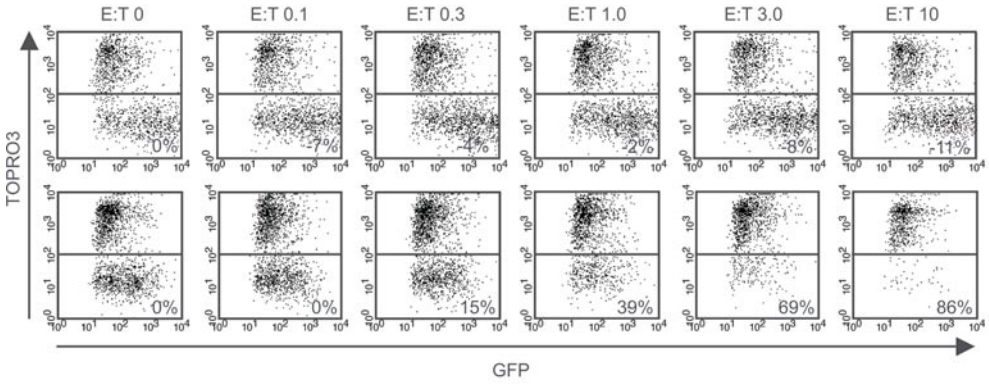


Figure 6. Detection of target cell elimination in the FATT-CTL assay. HLA-B*3501-positive BLCL were transfected with plasmid expressing only GFP (upper panels) or GFP-NP ESV (lower panels) and were used as target cells for CTL specific for the HLA-B*3501-restricted $NP_{418-426}$ epitope. Effector cells were added at different E:T ratios as indicated. The elimination of viable (TOPRO-3 negative) cells in the GFP-positive gated events was measured. The percentages indicate the proportion eliminated GFP^+ cells.

NP-specific killing of target cells *in vitro* is reduced by the loss of the $NP_{418-426}$ epitope

Next, MHC class I-matched BLCL were transfected with plasmid expressing GFP-NP with the ESV, DPI or DRI variant of the $NP_{418-426}$ epitope and were incubated with CTL clones specific for the conserved NP_{44-52} epitope or CTL clones specific for the $NP_{418-426}$ ESV or DPI epitope. As shown in figure 7, both $NP_{418-426}$-specific CTL clones lysed BLCL transfected with plasmids expressing the corresponding homologous $NP_{418-426}$ epitope variants and failed to lyse BLCL expressing the NP with the DRI variant of the $NP_{418-426}$ epitope, whereas the control NP_{44-52}-specific CTL clone lysed BLCL transfected with each of the variant GFP-NP plasmids (figure 7A-C). To study the impact of the loss of the $NP_{418-426}$ epitope on the lytic activity of polyclonal virus-specific $CD8^+$ T-cell populations, PBMC of the HLA-B*3501-positive study subjects 3180, 6358 and 0775 were stimulated with influenza viruses A/NL/95-ESV, -DPI and -DRI. The highest lytic activity was observed against cells transfected with plasmid GFP-NP ESV in polyclonal $CD8^+$ T-cell populations stimulated with influenza virus A/NL/95-ESV (student t-test, $p<0.05$). The lytic activity in these cultures against cells transfected with plasmids GFP-NP DPI and -DRI was comparable to that observed in PBMC cultures stimulated with influenza viruses A/NL/95-DPI and -DRI. In all of the three study subjects tested, similar lytic activity was observed as shown in figure 7D-F.

Discussion

In the present study, we examined the effect of the loss of the hypervariable $NP_{418-426}$ epitope on the human influenza virus-specific CTL response *in vitro*. The loss of this epitope affect-

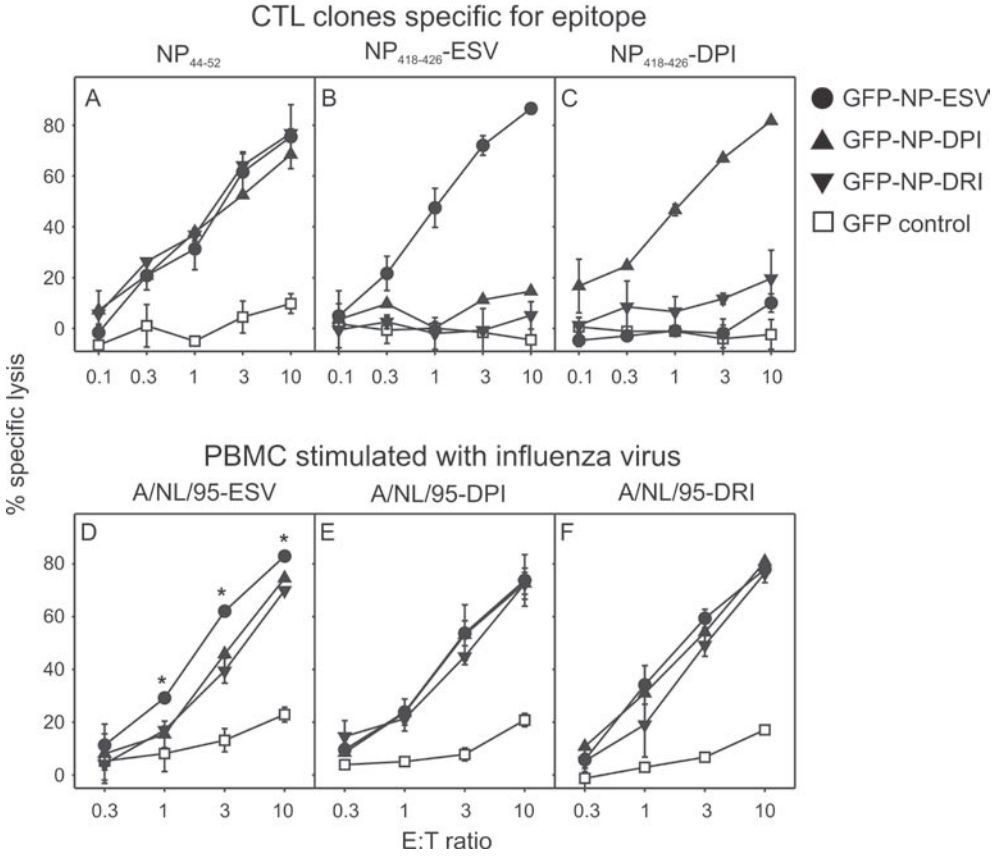


Figure 7. Amino acid variation in the $NP_{418-426}$ epitope affects the lytic activity of CTL *in vitro*. HLA-A*0101- and -B*3501-positive BLCL were transfected with plasmid expressing only GFP (□), GFP-NP ESV (●), -DPI (▲) or -DRI (▼) and were used as target cells for CTL clones specific for the HLA-A*0101-restricted NP_{44-52} epitope (A) or the HLA-B*3501-restricted $NP_{418-426}$ epitopes ESV (B) or DPI (C), or as target cells for $CD8^+$ cells isolated from PBMC cultures stimulated with influenza virus A/NL/95-ESV (D), -DPI (E) or -DRI (F) in the FATT-CTL assay. Effector cells were added at different E:T ratios as indicated, and percentages specific lysis were calculated. The error bars indicate the standard deviation. The asterisks indicate statistical significance (student t-test, $p<0.05$). For graphs D-F, the result of a representative experiment is shown (study subject 6358).

ed the virus-specific CTL response of HLA-B*3501-positive study subjects significantly, resulting in reduced IFN-γ production and lytic activity.

In order to study CTL responses against influenza viruses with or without the $NP_{418-426}$ epitope *in vitro*, three identical recombinant influenza viruses were generated only differing in their $NP_{418-426}$ amino acid sequence. We used the epitope sequence of influenza virus A/PR/8/34 (LPFDRTTIM) and the epitope sequence of the virus-variant that circulated between 1957 and 1972 (LPFDKPTIM) for the generation of viruses without the $NP_{418-426}$ epitope, since these epitope variants are not recognized by CTL specific for the contemporary variant of the epitope (LPFEKSTVM) (26, 27). This was confirmed by showing that MHC class I-matched BLCL infected with influenza virus A/NL/95-DRI were not recog-

nized by clonal or polyclonal CTL populations specific for the $NP_{418-426}$ ESV variant of the epitope (figures 1 and 3). The introduction of historic influenza virus $NP_{418-426}$ amino acid sequences into a more recent influenza virus-backbone did not affect viral fitness, since all these recombinant viruses were rescued by reverse genetics technology and replicated to similar extents. In addition, infection of BLCL, antigen processing and presentation was not affected by amino acid substitutions in the $NP_{418-426}$ epitope since CTL specific for the conserved NP_{44-52} and $M1_{58-66}$ epitopes recognized MHC class I-matched BLCL infected with the three different $NP_{418-426}$ variant viruses equally well.

Stimulation of PBMC obtained from HLA-B*3501-positive subjects with the variant viruses resulted in the expansion of $NP_{418-426}$-specific $CD8^+$ T-lymphocytes that in general were only detected with tetramers containing the homologous peptides. Thus, both with clonal and polyclonal $NP_{418-426}$-specific T-cell populations, no or very little cross-reactivity was observed with heterologous peptide variants, which is in accordance with previous findings (26, 27), and which justified the use of these $NP_{418-426}$-variant sequences as epitopes no longer recognized by ESV-specific CTL. Indeed, ESV-specific $CD8^+$ T-cells that were detected in four out of the six subjects studied did not cross-react with the two peptide-variants. In one of the two other subjects, a response was detected against the DPI-variant epitope that was present in viruses circulating between 1957 and 1972. This response cross-reacted to some extent with the ESV-variant. The differences in $NP_{418-426}$-response most likely represent differences in history of infection and exposure to the various variants (27). In none of the PBMC cultures stimulated with influenza virus with the DRI-variant of the epitope, CTL were detected reactive with the ESV- or the DPI-variant. The data obtained with tetramer staining were confirmed by intracellular IFN-γ staining upon re-stimulation with the respective peptide variants. The latter assay was also used to assess the frequency of virus-specific $CD8^+$ T-cells in the PBMC expansion cultures stimulated with influenza viruses A/NL/95-ESV, A/NL95-DPI and A/NL/95-DRI. The expanded T-lymphocytes were re-stimulated with all three viruses to induce IFN-γ expression, and the frequency of virus-specific CTL re-stimulated with influenza viruses A/NL/95-ESV and A/NL95-DPI were compared with those re-stimulated with influenza virus A/NL/95-DRI. In the $CD8^+$ T-lymphocyte populations expanded after stimulation with influenza virus A/NL/95-DRI, the frequency of IFN-γ^+ cells after re-stimulation with influenza viruses A/NL/95-ESV, A/NL95-DPI or A/NL/95-DRI was very similar. However, in influenza virus A/NL/95-ESV stimulated T-cell populations a statistical significant reduction was observed after re-stimulation with influenza virus A/NL/95-DRI, especially in the study subjects with an appreciable response to the ESV-variant of the epitope. In these subjects the reduction accounted for 20-32% of the overall influenza virus-specific CTL response. A similar reduction of the *in vitro* CTL response was observed after the loss of the HLA-B*2705-restricted $NP_{383-391}$ epitope (16). Thus, the loss of a single epitope can have a significant effect on the virus-specific CTL response *in vitro*. Next, we wished to determine whether the reduction of the IFN-

γ response correlated with a reduction in lytic activity of these virus-specific CTL, since the elimination of virus-infected cells is considered to be important for the viral clearance and control of the infection *in vivo* (179, 248). The NP is a major target for the human influenza virus-specific CTL response (94) and most CTL epitopes that have been identified are located in this protein (71, 185). Therefore, we measured NP-specific lytic activity of the *in vitro* expanded polyclonal T-cell populations in the FATT-CTL assay, using plasmids from which the wild type and mutant NP genes are expressed as GFP-fusion proteins. In contrast to the traditional chromium-release assay, the FATT-CTL assay allows the identification of protein expressing target cells, which increases the sensitivity of the assay and the window of opportunities to measure small differences in lytic activity of T-cell populations (249). Using influenza virus-specific CTL clones, the FATT-CTL assay was evaluated for the detection of lytic activity specific for the $NP_{418-426}$ and the $NP_{383-391}$ epitope. The specificity of the lytic activity was confirmed with $NP_{418-426}$-specific CTL clones, since only cells were lysed that were transfected with the NP gene encoding the homologous epitope sequence. Similar results were obtained with the $NP_{383-391}$ specific CTL clone (data not shown). Next, we assessed the effect of variation in epitope sequences on the NP-specific lytic activity of polyclonal CTL responses *in vitro*. Amino acid substitutions at the TCR contact residues of the $NP_{418-426}$ epitope reduced the lytic activity of CTL obtained from HLA-B*3501-positive individuals significantly. Similar results were obtained with influenza viruses with and without the $NP_{383-391}$ epitope and polyclonal virus-specific CTL populations (data not shown). Thus, both amino acid substitutions at TCR contact residues and anchor residues can result in the impairment of the lytic activity of the CTL response. During *chronic* virus-infections a potent and focused $CD8^+$ T-cell response is correlated with a strong decline in virus-titers (138). However, the emergence of amino acid substitutions within immunodominant epitopes can diminish CTL recognition resulting in loss of control of virus-replication. The mutant virus becomes predominant and the wild type virus eliminated, as a result of intra-host selective pressure by virus-specific CTL. Since influenza A viruses cause *acute* infections, the selective pressure within individual patients may be limited. However, influenza viruses are present in the human population persistently, causing worldwide epidemics annually. Therefore, selective pressure by CTL on influenza viruses might take place at the population level (88). As expected, the reduction of lytic capacity of the CTL response correlated with a reduction in the number of virus-specific $CD8^+$ T-cells that could respond. Since the epitope variants with mutations at TCR contact residues may lead to novel virus-specific CTL responses in the population with specificity for the mutant epitope, the immune escape could be only temporary, and the immune pressure on the epitope could continue to exist and may drive further diversification. This of course, is not the case after loss of the epitope by an amino acid substitution at anchor residues. Although it has been possible with the FATT-CTL assay to measure lytic activity against HIV-1 in PBMC *ex vivo* without prior expansion of virus-specific CTL (249), this was not possible for influenza virus-specific CTL (data not shown).

Most likely, the number of virus-specific CTL in chronically infected HIV-1 patients is much higher and they may have the effector-phenotype, unlike the influenza virus-specific memory cells in the peripheral blood (54, 205).

Collectively, the data show that the loss of, or the variation in individual CTL epitopes can affect the influenza A virus-specific immune response significantly. It not only results in a reduction of the number of virus-specific $CD8^+$ T-cells that can respond to produce cytokines, like IFN-γ, but it also impaired the lytic activity towards infected cells. This in turn may result in delayed clearance of influenza virus infection accompanied with more severe disease and increased mortality rates, as was also shown in C57BL/6 mice infected with a virus from which the immunodominant H-$2D^b$-restricted $NP_{366-374}$ epitope was deleted (259). Prolonged viral shedding of mutant virus in selected MHC class I-matched individuals in the human population also could explain the rapid fixation of the mutations in a theoretical model (88). Their high mutation rate and adaptive nature allow influenza viruses to evade both humoral and cell-mediated host immunity, which may contribute to their persistence in the human population. Furthermore, the variability in CTL epitopes may have implications for the development of vaccines, which also aim at the induction of CTL responses. The use of influenza virus A/PR/8/34 as vaccine-backbone strain or other influenza virus strains (e.g. the cold adapted strains) of which CTL epitope sequences do not fully match with current epidemic strains or future heterosubtypic pandemic strains may lead to suboptimal CTL responses, which in turn may affect vaccine effectiveness.

Acknowledgements

This work was financially supported by European Union grant QLRT-2001-01034 (Novaflu). We thank T.M. Bestebroer for excellent technical assistance, and B. Tomson and Y. Kruyt for the selection of HLA-typed blood donors (Sanquin Bloodbank).

Chapter

8

An amino acid substitution in the influenza A virus hemagglutinin associated with escape from recognition by human virus-specific $CD4^+$ T-cells

E.G.M. Berkhoff • M.M. Geelhoed-Mieras • M. Jonges • D.J. Smith
R.A.M. Fouchier • A.D.M.E. Osterhaus • G.F. Rimmelzwaan

Department of Virology and postgraduate school Molecular Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.
Department of Zoology, Cambridge University, Cambridge, UK

Submitted for publication

Influenza virus-specific $CD4^+$ T-helper cells were cloned that recognized a virus strain isolated in 1981, but that failed to recognize more recent strains. The HLA-DR*1601-restricted epitope recognized was located in the hemagglutinin (HA_{99-113}) and the naturally occurring A→V substitution at position 106 was responsible for abrogating the recognition by HA_{99-113}-specific $CD4^+$ T-cells. This amino acid substitution was found in influenza A/H3N2 viruses that circulated between 1999 and 2005 and did not affect recognition by virus-specific antibodies. It was speculated that influenza viruses could evade recognition by virus-specific $CD4^+$ T-cells, at least temporarily.

In response to influenza virus infection, a variety of host immune responses is induced, like the production of virus-neutralizing antibodies by B-cells and the activation of virus-specific $CD8^+$ cytotoxic T-lymphocytes (CTL) that can eliminate virus-infected cells (60, 110). Virus-specific $CD4^+$ T-helper cells promote and regulate these responses (11, 31, 144, 207, 239). Influenza viruses are genetically instable and as a result variants are selected that can evade host immunity, which is induced by annual epidemics of influenza virus infections. Extensive antigenic changes in the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins caused by the accumulation of amino acid substitutions, also known as antigenic drift, can abrogate binding and the neutralizing effect of antibodies (274). More recently, it was shown that influenza A viruses can also evade cell-mediated immunity. The accumulation of amino acid substitutions in influenza virus CTL epitopes can affect binding of immunogenic peptides to MHC class I-molecules or diminish T-cell receptor interaction, preventing recognition by virus-specific CTL (26, 202, 211, 253). The loss of a single epitope affected the human influenza virus-specific $CD8^+$ T-cell response *in vitro* significantly, as measured by IFN-γ production and lytic activity (16, 18). The reduced CTL responses may result in prolonged infection and viral shedding, and possibly affects disease outcome (169). To date, immune evasion from $CD4^+$ T-cells by influenza A viruses has not been described. In the present study, we describe an amino acid substitution in the HA of influenza A/H3N2 viruses that is associated with the abrogation of recognition by virus-specific $CD4^+$ T-cells.

To obtain T-cell clones that recognize variable epitopes, peripheral blood mononuclear cells (PBMC) of a healthy blood donor (MHC class II-genotype DR*1303, DR*1601, DQ*0301, DQ*0502, DP*0401), 46 years of age, were stimulated with influenza A/H3N2 virus that was isolated in 1981 (A/NL/4791/81), as previously described (19, 25). After expansion of virus-specific T-cells, they were cloned by limiting dilution and further expanded by several rounds of non-specific stimulation using phytohemagglutinin (253). A total of 26 $CD4^+$ T-cell clones were obtained that specifically recognized autologous B-lymphoblastoid cell line (BLCL) infected with influenza virus A/NL/4791/81, as was determined by IFN-γ ELISpot assays, which were performed according to the instructions of the manufacturer

(Mabtech, Stockholm, Sweden). Two of the 26 $CD4^+$ T-cell clones failed to recognize BLCL infected with an H3N2 virus isolated in 2003 (A/NL/9/03), indicating that these two $CD4^+$ T-cell clones recognize variable epitopes.

The MHC class II-restriction element recognized by the two $CD4^+$ T-cell clones was determined, using partially HLA-matched BLCL (genotypes: **DR*1601**, **DQ*0502, DP*0401**, DP*1401**;** DR*1202, **DQ*0301**, **DQ*0502**, DP*1301, DP*2101**;** DR*1501, DR*1201, **DQ*0301**, DQ*0601**;** DR*0301, **DR*1301**, DQ*0101, DQ*0603**;** **DR*1302**, DR*0802, DQ*0402, DQ*0604**;** DR*0401, DR*0406, **DQ*0301**, DQ*0402, **DP*0401;** matching genotypes are depicted in bold) infected with influenza virus A/NL/4791/81 for the stimulation of the two $CD4^+$ T-cell clones in IFN-γ ELISpot assays. Since only the autologous and the first BLCL listed above where recognized by the two $CD4^+$ T-cell clones in IFN-γ ELISpot assays, the MHC class II-restriction element was determined as HLA-DR*1601. For the identification of the viral protein recognized by the two $CD4^+$ T-cell clones, autologous BLCL were transfected with plasmids expressing individual influenza virus genes, using nucleofector technology according to the instructions of the manufacturer (Amaxa Biosystems, Cologne, Germany). The transfected cells were used for the stimulation of the two $CD4^+$ T-cell clones and induction of IFN-γ expression was tested by ELISpot assays. It was found that the two $CD4^+$ T-cell clones were specific for HA. Generation of the plasmids was described previously (17). Next, the location of the epitope recognized by the two $CD4^+$ T-cell clones in the HA and the mutations responsible for abrogation of this recognition were determined, using an epitope prediction algorithm (http://www.epipredict.de) and the recognition pattern of BLCL infected with various influenza viruses for which HA amino acid sequences were available (influenza viruses A/NL/4971/81, A/Hong Kong/1/68, A/Nanchang/933/95, A/NL/178/95, A/NL/462/98 and A/NL/22/03 were recognized by the two $CD4^+$ T-cell clones, whereas influenza viruses A/NL/9/03, A/NL/120/02 and A/NL/20/03 were not recognized by the two $CD4^+$ T-cell clones). The predicted $HA_{99\text{-}113}$ amino acid sequence (PYDVPDYASLRSLVA) matched the results and an amino acid substitution at position 106 (A106V) (position 1 being the first amino acid of HA1) was identified as the mutation responsible for the abrogation of $CD4^+$ T-cell recognition. Synthetic peptides with and without the A106V-substitution were manufactured, HPLC-purified and analyzed with mass spectrometry (Eurogentec, Seraing, Belgium). In addition, recombinant influenza viruses with and without the HA A106V-substitution were generated using a bidirectional reverse genetics system based on the PB2, PB1, PA, NP, M and NS gene segments of influenza virus A/Puerto Rico/8/34, and the HA and NA gene segments of influenza virus A/NL/178/95 (H3N2) or A/Netherlands/213/03 (A/NL/213/03; H3N2) (58, 114). Both viruses had an alanine at position 106 of the HA, and by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA) the alanine at this position was substituted for a valine. To verify that viral fitness was not affected by this substitution, multi-step growth curves were determined in Madin-Darby Canine Kidney (MDCK)

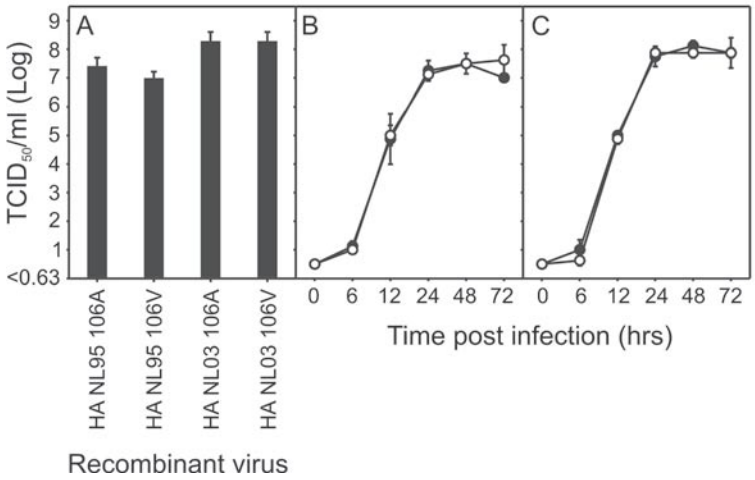


Figure 1. Growth kinetics and viral fitness were not affected by the HA A106V-substitution. Upon transfection of 293T cells and subsequent rescue in MDCK cells, infectious virus titers were determined for the influenza viruses with and without the HA A106V-substitution (A). Subsequently, growth curves were generated after infection of MDCK cells at a MOI of 0.001 (B and C). Virus replication kinetics for recombinant influenza virus A/NL/178/95 (B) and A/NL/213/03 (C) with (○) and without (●) the HA A106V-substitution are shown. The data represent the average of two experiments. The error bars indicate the standard deviation.

cells, as described previously (17). The viruses with or without the HA A106V-substitution replicated to similar titers and also the kinetics of virus replication was similar (figure 1). Before $CD4^+$ T-cell recognition experiments were performed with these viruses it was confirmed that viruses with and without the A106V-substitution infected BLCL equally well as determined by immunofluorescence assays using a FITC-conjugated monoclonal antibody (DAKO, Glostrup, Denmark) directed to the viral nucleoprotein. Next, autologous BLCL were infected with the recombinant viruses and used for the re-stimulation of the T-cell clones specific for the variable $HA_{99\text{-}113}$ epitope and for a $CD4^+$ clone specific for a conserved epitope. As shown in figure 2, the $HA_{99\text{-}113}$-specific $CD4^+$ T-cell clone recognized stimulator cells infected with viruses with the wild type HA-sequence, but failed to recognize autologous BLCL infected with mutant viruses containing the A106V-substitution. These results were confirmed with peptide-pulsed BLCL. In contrast, a $CD4^+$ T-cell clone specific for a conserved epitope recognized the autologous stimulator cells regardless which virus was used for infection, and failed to recognize the $HA_{99\text{-}113}$ peptide. In order to determine to which extent the $HA_{99\text{-}113}$-specific response contributed to the virus-specific response, the PBMC of four HLA-DR*1601-positive study subjects were stimulated with recombinant influenza viruses containing the wild-type epitope for ten days. Subsequently, the $CD4^+$ cells were isolated using CD4-microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) and re-stimulated with autologous BLCL infected with recombinant influenza virus or pulsed with the wild type $HA_{99\text{-}113}$ peptide. The frequency of cells producing IFN-γ was assessed in ELISpot assays (table 1). The proportion of $HA_{99\text{-}113}$ (106A)-specific cells

Table 1. Contribution of HA_{99-113} specific $CD4^+$ T-cells to virus-specific response

Subject	number of $CD4^+$ T-cells/10^3 cells specific for:		Relative proportion of HA_{99-113}
	HA NL03 106A	HA_{99-113}	
6104	266	18	6.8 %
3569	102	5	4.9 %
1524	102	2	2.0 %
1434	114	7	6.1 %

The number of specific $CD4^+$ T-cells was determined by IFN-γ-specific ELISPOT assays after *in vitro* re-stimulation with autologous BLCL infected with recombinant influenza virus A/NL/213/03 106A or pulsed with 10 μM HA_{99-113} peptide. The relative proportion of HA_{99-113}-specific $CD4^+$ T-cells was calculated as follows: 100% × number HA_{99-113}-specific spots per 10^3 cells / number virus-specific spots per 10^3 cells. The averages of two independently repeated experiments are given.

constituted 2.0 – 6.8% of all virus-specific $CD4^+$ T-cells, indicating that the HA_{99-113}-specific response was not immunodominant in the HLA-DR*1601-positive study subjects. Stimulation of PBMC with non-recombinant influenza virus A/NL/213/03 yielded similar results, indicating that the A/PR/8/34 background in the recombinant viruses did not result in an underestimation of the number of virus-specific $CD4^+$ T-cells. In polyclonal $CD4^+$ T-cell populations obtained after stimulation with influenza virus A/NL/03-106A, the frequency of cells responding to wild type peptide was higher compared to that responding to the mutant peptide (figure 2D). In contrast, re-stimulation with influenza virus A/NL/03-106A did not result in significant higher number of responding cells compared to re-stimulation with A/NL/03-106V, which is in agreement with the subdominant nature of the HA_{99-113} epitope.

Since virus-neutralizing antibodies exert considerable selective pressure on the HA-molecule, we wished to exclude an effect of the HA A106V-substitution on antibody recognition. The A106V-substitution was not characteristic for any of the cluster transitions in the antigenic evolution of influenza A(H3N2) viruses from 1968 to 2003 (227). In addition, the effect of the A106V-substitution on antibody recognition was tested in hemagglutination-inhibition (HI) assays (208), using recombinant influenza viruses A/NL/178/95 and A/NL/213/03 with and without the A106V-substitution in addition to a panel of reference viruses and post-infection ferret sera against them. The antibody titers obtained in the HI-assay were used to construct an antigenic map, as described recently (227). As shown in figure 2E, each of the viruses within a pair was antigenically very similar (less than one unit of antigenic distance, which is the difference between grid lines in figure 2E and corresponds to a 2-fold difference in HI-titer). These data indicate that virus-specific antibodies did not select for the HA A106V-substitution, since a correlation with antigenic drift could not be demonstrated.

To study the evolution of the HA_{99-113} epitope over time, we examined the HA amino acid sequence of 2869 influenza A/H3N2 viruses isolated between 1968 and 2006 that were submitted to the Influenza Sequence Database at www.flu.lanl.gov (158). Before 1983, all viruses contained an alanine at position 106. In 1983/1984, two out of twenty three viruses

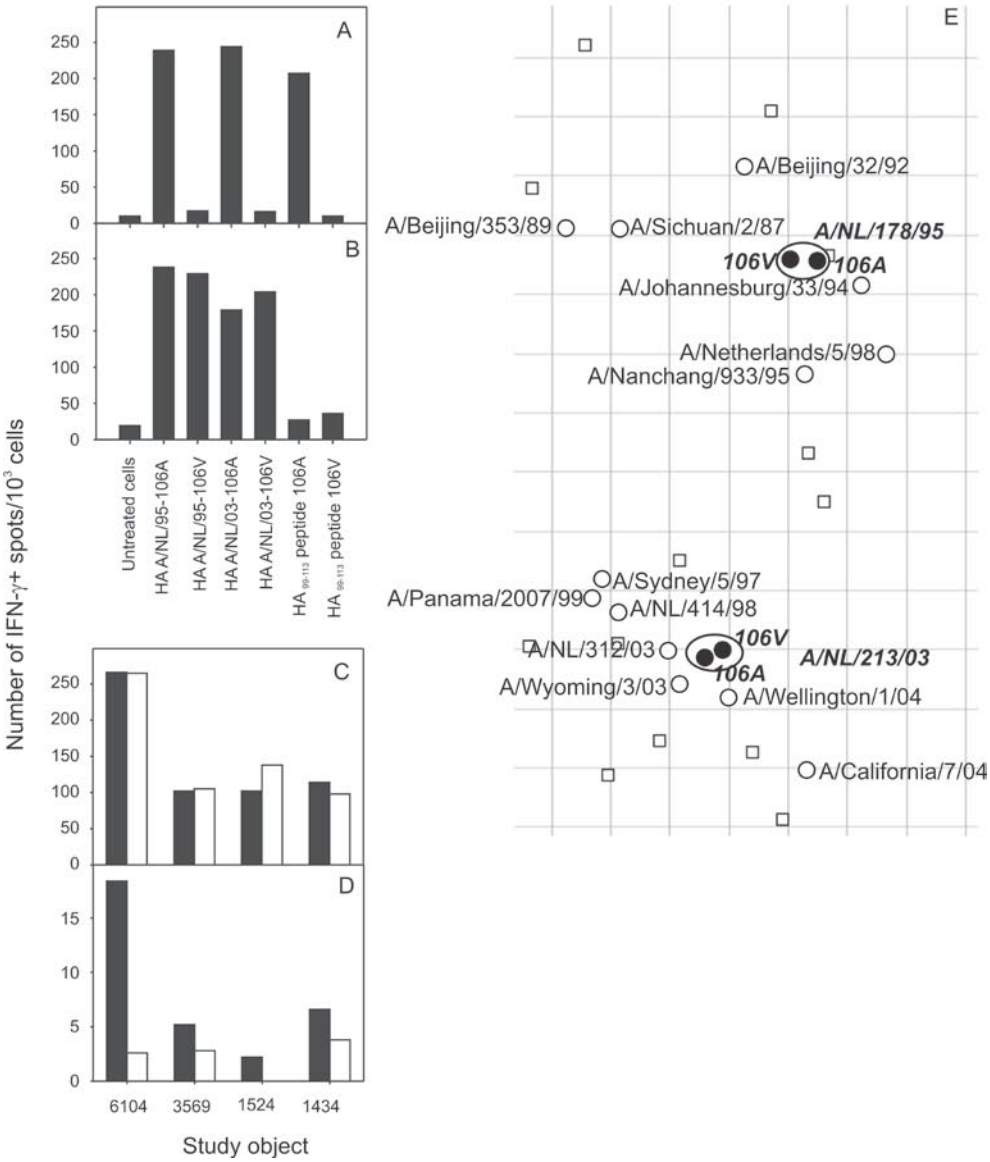


Figure 2. Recognition of peptide-pulsed or virus-infected BLCL by clonal and polyclonal virus-specific CD4$^+$ T-cells, and the comparison of the antigenic properties of the recombinant viruses with and without the A106V-substitution. An $HA_{99\text{-}113}$ (PYDVPDYASLRSLVA)-specific CD4$^+$ T-cell clone (A) and a CD4$^+$ T-cell clone specific for a conserved epitope (B) were stimulated with BLCL infected with recombinant viruses A/NL/178/95 (HA NL95) or A/NL/213/03 (HA NL03) with or without the A106V-substitution or pulsed with wild type or mutant $HA_{99\text{-}113}$ peptide as indicated, and the number of cells producing IFN-γ was determined in ELISpot assays. Untreated cells were included as a negative control. Five hundred effector cells were incubated with 2.5×10^4 stimulator cells. The data of a representative experiment is shown, and similar results were obtained for the other $HA_{99\text{-}113}$ (PYDVPDYASLRSLVA)-specific CD4$^+$ T-cell clone. In addition, polyclonal CD4$^+$ T-cells expanded after stimulation of PBMC with HA A/NL/03-106A were (C) re-stimulated with autologous BLCL infected with influenza viruses A/NL/03-106A (black bars) and A/NL/03-106V (open bars) or (D) pulsed with wild type peptide (black bars) or mutant peptide (open bars). The average values of two independently repeated experiments are shown after subtraction of background values obtained after stimulation with untreated cells. To

assess the effect of the A106V-substitution on the antigenic property of the HA-molecule, influenza viruses A/NL/178/95 and A/NL/213/03 with and without the A106V-substitution were tested in the HI-assay together with a number of reference strains (as indicated in figure 2E) and post infection ferret sera raised against them. Distances were calculated from the HI-titers and the strains and sera were positioned in a map by modification of metric multidimensional scaling. The spacing between the grid lines is one unit of antigenic distance, corresponding to a two-fold dilution of antiserum in the HI-assay. Two units correspond to fourfold dilution, three units to eightfold dilution, etcetera. Depicted are the relative positions of reference influenza virus strains (open circles), post-infection ferret sera (open squares), and the two pairs of recombinant viruses with and without the A106V-substitution (black circles).

contained the A106V-substitution. Also in 1993, the substitution was sporadically detected (3/152). However, between 1999 and 2005 there was a surge of viruses containing the mutation with a peak in 2002, when 202 out of 296 viruses (68%) contained the A106V-substitution. For 2006, no 106V HA-sequences could be found in the Influenza Sequence Database (figure 3). Consultation of the Influenza Sequence Database (158) did not reveal any co-mutations in HA uniquely associated with the A106V-substitution, excluding the possibility that the A106V-substitution is the result of "hitch-hiking" on other mutations important for viral fitness. In addition, it has been shown that mutations in the influenza virus HA are not linked to mutations in its internal genes (155).

Thus, the A106V-substitution in the HA-molecule failed to reach fixation, unlike substitutions in some CTL epitopes (26, 88). It is possible that the A106V-substitution resulted in some temporarily selective advantage at the population level, although in HLA-DR*1601-positive individuals the A106V-substitution did not affect the *in vitro* virus-specific T-helper cell response significantly. The selective advantage may not have been sufficient to drive fixation and the surge of mutant viruses came to an end in 2006. Possibly, HA-106A viruses had a higher intrinsic viral fitness (other than replication kinetics) that was more important than the immune evading properties of the 106V mutant-sequence. Alternatively, viruses with another, stronger selective advantage containing an A at position 106 prevailed. In this case, the revertance to 106A not necessarily contributed to the selected advantage as for example was also found for viruses containing a mutation in the M2 gene conferring resistance to amantadine, which is hardly used and which cannot be responsible for significant selective pressure (Holmes, personal communication). Both hypotheses are in agreement with the subdominant nature of the $HA_{99\text{-}113}$ -specific $CD4^+$ T-cell response in HLA-DR*1601-positive individuals (table 1) and the low prevalence of HLA-DR*1601 in the human population, which ranges from 0-18% depending on the geographical and ethnical distribution (163). When immune pressure would have been more robust, the selective pressure results in the fixation of the mutation as was found for antibody-driven variation (227) and for variation in epitopes recognized by virus-specific CTL (88, 253).

Escape from $CD4^+$ T-cells has been demonstrated in $CD4^+$ TCR-transgenic mice specific for an immunodominant MHC class II-restricted lymphocytic choriomeningitis virus epitope (45). In addition, $CD4^+$ T-cell escape has been observed in human immunodeficiency virus positive individuals (105) and hepatitis C virus immunized chimpanzees (203). In the latter, viral escape from $CD4^+$ T-cells resulted in the eventual failure of a T-cell

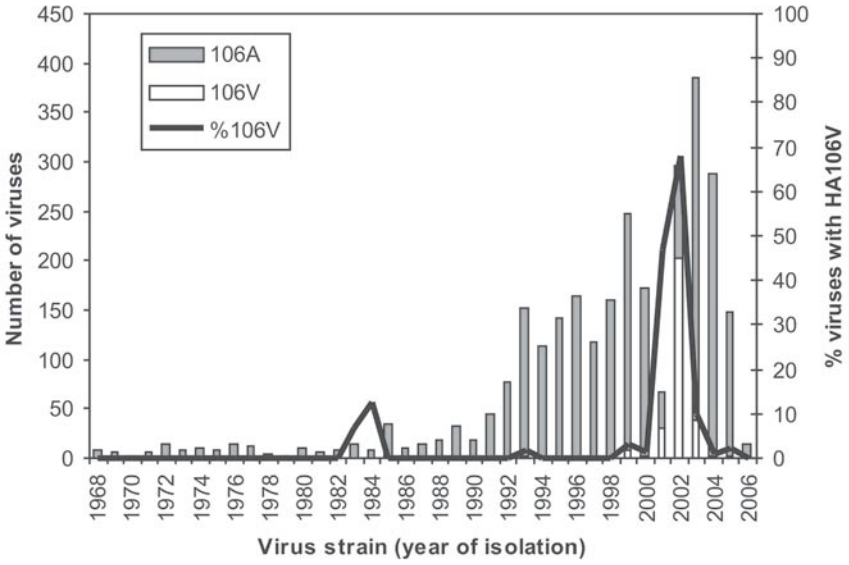


Figure 3. The prevalence of the $HA_{99\text{-}113}$ epitope variants during influenza A (H3N2) virus evolution. To study the evolution of the $HA_{99\text{-}113}$ epitope over time, we examined the HA amino acid sequence of 2869 influenza A/H3N2 viruses isolated between 1968 and 2006 that were submitted to the Influenza Sequence Database at www.flu.lanl.gov (158). Indicated are the total numbers of viruses containing the 106A amino acid sequence (gray bars) and the 106V epitope amino acid sequence (white bars) per year, and the percentage viruses containing the 106V amino acid sequence (line).

response that initially controlled infection. At this point it is unclear how the (temporary) escape from $CD4^+$ T-cells contributed to improved immune fitness of influenza A/H3N2 viruses. The loss of the T-helper cell epitope may have affected antibody or CTL responses. Alternatively, it could have affected the anti-viral activity of the $CD4^+$ response more directly, since $CD4^+$ T-cells also possess cytolytic activity (30, 64, 115, 217).

Collectively, variation in the influenza virus hemagglutinin was identified not associated with escape from recognition by antibodies but that prevented recognition by T-helper cells specific for the HLA-DR*1601-restricted $HA_{99\text{-}113}$ epitope. Since this epitope was subdominant, a difference in T-helper cell responses to virus with and without the epitope was not detected in HLA-DR*1601-positive individuals. To obtain full understanding of the contribution of influenza virus-specific T-helper cell responses to the virus' evolution and dynamics in the population, more research is required.

Acknowledgements

This work was supported by European Union grant QLRT-2001-01034 (Novaflu). DJS was supported by NIH Director's Pioneer Award Program through grant number DP1-OD000490-01. We thank B. Tomson and Y. Kruyt for the selection of HLA-typed blood donors (Sanquin Bloodbank).

Chapter

9

Summarizing discussion

The studies presented in this thesis describe the impact of the loss of influenza virus epitopes on the human virus-specific CTL response *in vitro*, the extent of variation in T-cell epitopes and the effect of amino acid substitutions in CTL epitopes on viral fitness.

CTL can eliminate infected cells directly and rapidly (128, 247), and therefore play an important role in the control of viral infections, including those caused by influenza viruses. Studies in mice have shown that upon adoptive transfer of virus-specific CTL, mortality caused by influenza virus infection was reduced and more efficient viral clearance was observed (159, 237, 267, 276, 277). In addition, mice lacking $CD8^+$ T-cells showed increased mortality and delayed viral clearance (12, 222, 266). Also in humans, the level of influenza virus-specific CTL activity correlated with the rate of viral clearance upon infection (168, 169). Influenza virus-specific CTL are not only protective against drift variants within a subtype, but also against heterosubtypic virus strains, as has been shown in animals (14, 44, 73, 140, 239) and humans (125, 168). Records from the 1957 pandemic showed that adults were three times less susceptible to influenza virus H2N2 infection than children, whereas no significant difference in infection was observed in the preceding epidemics (70). Protection by cross-reactive CTL may explain why the 1957, 1968 and 1977 pandemics were relatively moderate. Also during the current influenza virus H5N1 outbreaks, more morbidity and mortality in children and young adults is reported than in older individuals, which may have been exposed to human influenza virus more frequently, and have developed cross-reactive CTL memory.

However, certain viruses can escape from specific CTL by accumulating amino acid substitutions at sites important for epitope processing, MHC class I-binding, and/or TCR recognition (138, 183, 197). Also influenza viruses can evade CTL responses by amino acid substitutions in CTL epitopes (26, 185, 202, 253). The R384G substitution in the influenza virus NP is at the anchor residue of the HLA-B*0801-restricted $NP_{380-388}$ epitope and the HLA-B*2705-restricted $NP_{383-391}$ epitope, and prevented binding of the epitopes to their corresponding MHC class I-molecules and recognition by specific CTL (253). Amino acid variation at positions 421, 422, 423, and 425 of the NP, which form the TCR contact residues of the HLA-B*3501-restricted $NP_{418-426}$ epitope, also resulted in evasion from specific CTL. Influenza viruses with variation in the $NP_{418-426}$ epitope emerged over time, and $NP_{418-426}$ specific CTL directed against earlier virus strains failed to recognize more recent variants of influenza virus, indicating escape from CTL immunity (26). For some $NP_{418-426}$ epitope variants, a small proportion of the CTL directed against one epitope variant cross-reacted with another epitope variant (27). In a subsequent infection with an influenza virus with the corresponding variant epitope these cross-reactive CTL may be further expanded, indicating that immune evasion may only be temporary.

Since it was unclear to what extent amino acid substitutions in CTL epitopes affect the virus-specific CTL response, we assessed the effect of the loss of influenza virus epitopes on the human influenza A virus-specific CTL response *in vitro*. We used *in vitro*

expanded virus-specific PBMC cultures, since the number of influenza virus-specific $CD8^+$ T-cells determined *ex vivo* in PBMC of healthy blood donors is only 0.1 to 0.5% of the total number of $CD8^+$ T-cells (25). The hierarchy of immunodominance (see below) of epitope-specific $CD8^+$ T-cells measured *ex vivo* correspond with those after *in vitro* stimulation (25), indicating that *in vitro* expansion of virus-specific CTL is non-biased. In addition, *in vitro* stimulation is necessary for the induction of the effector phenotype of the virus-specific $CD8^+$ T-cells. The PBMC used in our studies were obtained from healthy study subjects that were infected once or more with influenza A virus in the past, and therefore the T-lymphocytes are of a memory phenotype. There are two main subsets of memory T-cells, the effector-memory cells that are responsible for immediate protection against viral infection, and the central-memory cells that have not fully differentiated to the effector phenotype yet (148). Influenza virus-specific effector-memory T-cells can be found in the respiratory tract, which is the site of infection (250, 271). Most circulating influenza virus-specific $CD8^+$ T-cells have a central-memory T-cell phenotype (54, 205, 250). The use of PBMC as effector cells requires additional stimulation to induce the effector phenotype (103).

Using these *in vitro* stimulated PBMC cultures, we determined the virus-specific $CD8^+$ T-cell response directed against influenza virus with or without the $NP_{383-391}$ or $NP_{418-426}$ epitope by intracellular IFN-γ staining and flow cytometry. It was found that the loss of the HLA-B*2705-restricted $NP_{383-391}$ epitope or HLA-B*3501-restricted $NP_{418-426}$ epitope affected IFN-γ production by human influenza virus-specific CTL *in vitro* significantly (chapter 2 and 7). In addition, we determined if this significant reduction in IFN-γ production correlated with a reduction in lytic activity of these virus-specific CTL. For the measurement of the lytic activity of clonal and *in vitro* expanded polyclonal T-cell populations, we used classical chromium-release assays and the recently developed FATT-CTL assay, which is based on the use of target cells transfected with plasmids expressing viral proteins fused to GFP (249). In contrast to the classical chromium-release assays, the FATT-CTL assay allowed the detection of small differences in lytic activity of T-cell populations. A significant reduction of the NP-specific lytic activity of the human influenza virus-specific CTL response was detected, as a result of the loss of the HLA-B*2705-restricted $NP_{383-391}$ epitope and the HLA-B*3501-restricted $NP_{418-426}$ epitope (chapter 7). Thus, the loss of the $NP_{383-391}$ and the $NP_{418-426}$ epitope resulted in significant reduction of both IFN-γ production and lytic activity. Reduction in IFN-γ may affect immune regulatory activity as well as direct antiviral activity. The loss of lytic activity may result in delayed clearance of influenza virus infection, as was also shown in C57BL/6 mice infected with a virus from which the immunodominant $H\text{-}2D^b$-restricted $NP_{366-374}$ epitope was deleted (259), and may result in increased morbidity and mortality.

Although these studies clearly show that loss of a single CTL epitope can affect the influenza A virus response significantly *in vitro*, its significance *in vivo* remains to be elucidated. It has been argued that the immune response to influenza A virus is broad, multi-

specific and sufficiently plastic to contain virus replication of escape mutants. Indeed, many viral peptides have immunogenic potential (25, 87, 124), but only the epitopes that elicit high numbers of effective CTL play a role in the direct and rapid elimination of infected cells, which is referred to as immunodominance (282). Paradoxically, the existence of a broad and diverse repertoire of CTL epitopes, and the detection of a high percentage of CTL directed against a single epitope, are not mutually exclusive concepts (87), so that the loss of an immunodominant epitope can significantly affect the influenza virus response. Indeed, the immune response is sufficiently plastic to elicit antibodies and T-cells specific for new virus variants (26), but by the time they are generated, the virus already has caused prolonged virus infection associated with more severe disease and increased mortality. Upon a next encounter, the influenza virus again accumulates substitutions, which may lead to "antigenic drift" in sites important for both humoral and cell-mediated host immunity. Therefore, we argue that evasion from human T-cell immunity by influenza viruses could significantly contribute to influenza virus pathogenesis and persistence in the population.

For the generation of identical influenza viruses with and without the $NP_{383-391}$ or $NP_{418-426}$ epitope, we used reverse genetics technology. Since the next evading amino acid substitution within the $NP_{418-426}$ epitope could not be predicted, we used the historic epitope sequence of influenza virus A/PR/8/34 (LPFDRTTIM), isolated in 1934, and the sequence of a epitope variant that circulated from 1957 till 1972 (LPFDKPTIM) for the generation of viruses "without" the $NP_{418-426}$ epitope. Both amino acid sequences are not recognized by CTL specific for the current epitope variant (LPFEKSTVM) (26), and could therefore be used as "escape" mutants. In addition, we hypothesized that these natural sequences would not be detrimental to viral fitness and would yield viable viruses. Indeed all three influenza viruses could be rescued and replicated to a similar extent. The $NP_{418-426}$ variation neither affected infection, antigen processing or presentation by BLCL, since cells infected with the three variant viruses were all recognized equally well by CTL specific for the conserved NP_{44-52} and $M1_{58-66}$ epitopes. However, the introduction of a glycine (G) at position 384 of the NP of influenza virus A/Hong Kong/2/68 was detrimental to viral fitness, and we used the NP gene segment of influenza virus A/Netherlands/18/94 with and without the G384R reverse substitution to generate influenza viruses with and without the $NP_{383-391}$ epitope. The influenza virus sequence database at http://www.flu.lanl.gov (158) revealed seven amino acid co-mutations associated with the R384G substitution. We hypothesized that one or more of these co-mutations were functionally compensating for the detrimental effect of the R384G substitution. This hypothesis was tested with recombinant influenza viruses with the R384G substitution alone or in combination with one of the co-mutations. It was found that a second substitution at position 375 of the NP (E375G) partially restored viral fitness and nucleoprotein functionality (chapter 3), and that a third substitution in the NP (M239V) fully restored viral fitness of recombinant influenza viruses containing 375G and 384G (chapter 4). Similar findings have been observed for CTL escape mutants of HIV and SIV, which

also accumulated extra-epitopic co-mutations in the Gag-protein for restoration of viral fitness in the presence of mutations in CTL epitopes (84, 133, 189). The use of an analogous mechanism to restore viral fitness in the presence of escape mutations in different viruses underscores the significance of this mechanism in the replication of RNA viruses within the host. Viruses that are able to escape cell-mediated immunity may have a replicative advantage. This advantage is (partially) lost if the evasive amino acid substitutions affect viral fitness. Additional functionally compensating substitutions, like the extra-epitopic amino acid substitutions shown here, may restore intrinsic viral fitness (e.g. replication capacity), resulting in the successful evasion from T-cell immunity.

Thus, influenza viruses may need to overcome functional constraints to accumulate mutations in CTL epitopes and escape from CTL. The inability to overcome these functional constraints could explain the relatively conserved nature of most identified influenza virus CTL epitopes, limiting escape from CTL. In order to obtain an impression of the selective pressure mediated by virus-specific CTL, we performed a synonymous/non-synonymous (ds/dn) analysis with the NP nucleotide sequences of a pair of influenza A (H3N2) viruses consisting of influenza virus A/England/878/69, isolated shortly after the introduction of H3N2 virus in the human population, and a more recent strain, A/New York/12/2003. The NP gene was selected for this type of analysis since fourteen of the known epitopes are located within this protein. The ds/dn analysis revealed that in the ninety amino acids that constitute the fourteen known epitopes located in the NP, relatively more non-synonymous mutations occurred between 1969 and 2003 than in the rest of the protein, which is suggestive for a selective pressure. The HLA-B*3501-restricted $NP_{418-426}$ epitope constitutes the most variable amino acid sequence within the NP gene (71), and this hypervariable sequence had a major impact on the lower ds/dn ratio. The high degree of amino acid variability in the influenza virus NP epitopes is not linked to variation in the surface glycoproteins (155), and is probably driven by CTL that recognize their epitope with high affinity (24). Blast-search of up to 450 influenza virus H3N2 sequences indicated that all known CTL epitopes, including the hypervariable $NP_{418-426}$ epitope and the epitopes located within other viral proteins, retained their anchor residues with the exception of the HLA-B*0801-restricted $NP_{380-388}$ epitope and the HLA-B*2705-restricted $NP_{383-391}$ epitope, and five out of the fourteen partially overlapping epitopes were fully conserved despite the selective pressure. To determine if functional constraints were responsible for the virus' inability to accumulate amino acid substitutions in conserved epitopes, limiting immune escape from virus-specific CTL responses, we performed a mutational analysis of various epitopes and tested the effect of selected amino acid substitutions on viral fitness and immune recognition by CTL (chapter 5). Of special interest was the highly conserved immunodominant HLA-A*0201-restricted $M1_{58-66}$ (GILGFVFTL) epitope. Immune pressure by CTL against this epitope must be high, considering the immunodominant nature of the epitope (25, 231) and the high prevalence of HLA-A*0201 in the human population (163). Yet, the amino acid sequence of this epitope

is conserved, even between different subtypes of human influenza A virus. To address this issue, we performed alanine-replacements for each of the nine amino acid positions of this epitope, using reverse genetics. Whereas most alanine-replacements did not prevent rescue of recombinant influenza virus and were tolerated to variable extents, the replacement of the anchor residue at position 2 of the $M1_{58-66}$ epitope (I59A) was detrimental to viral fitness. The $M1_{58-66}$ epitope is located in the fourth N-terminal α-helix of the M1 protein, and mutations in this region may disturb the functional and structural integrity of the protein as has been described for mutations in the M1 helix six domain (35, 156), limiting escape from CTL. Some of the other alanine-replacements resulted in the partial loss of recognition by $M1_{58-66}$ specific CTL, but their impaired replication kinetics was not in favor of the emergence of these mutants. There may be a trade-off between intrinsic viral fitness and immune evasion, resulting in the survival of the fittest under the selective conditions.

Considering the dramatic effect of the alanine-replacement at the anchor residue, we decided to focus more closely on anchor-residues of this and other epitopes. To this end, conservative amino acid substitutions were introduced at the anchor residues of the HLA-A*0101-restricted epitopes $PB1_{591-599}$ and NP_{44-52}, the HLA-A*0201-restricted epitope $M1_{58-66}$, the HLA-B*2705-restricted epitope $NP_{174-184}$, and the HLA-B*3501-restricted epitope $NP_{418-426}$. Conservative amino acid substitutions at position 2 of the $M1_{58-66}$ epitope (M1 I59L and I59V) were less critical than the alanine-replacement, although the kinetics of viral replication was somewhat affected. However, the recombinant viruses with the M1 I59L or I59V substitution were fully recognized by $M1_{58-66}$ specific CTL, which makes it unlikely that these variants would emerge in the human population. Next, we evaluated the conservative anchor residues of the otherwise hypervariable epitope $NP_{418-426}$ (LPFEKSTVM). The relatively conservative amino acid substitutions at the anchor residue of the epitope were detrimental to viral fitness. Also conservative amino acid substitutions at the anchor residues of the epitopes $PB1_{591-599}$ (VSDGGPNLY), NP_{44-52} (CTELKLSDY) and $NP_{174-184}$ (RRSGAAGAAVK) affected viral fitness. The PB1 D593N substitution in particular was detrimental to viral fitness. Although the conservative NP E46Q substitution resulted in the loss of the anchor residue, which allowed the virus to escape from specific CTL, the loss of viral fitness may limit the emergence of this variant in the human population. We concluded that influenza A viruses display a limited degree of variation in CTL epitopes despite selective pressure on these epitopes mediated by CTL, which may be caused by functional constraints imposed on influenza virus CTL epitopes, limiting efficient escape from CTL. Similarly, fitness costs limit variation in the highly immunodominant Gag p11C, C-M CTL epitope of SIV and escape from specific CTL (190), and probably also plays a role in other viral infections.

Functional constraints may have limited the number of evading mutations in influenza A virus CTL epitopes. In addition, the use of old prototypic strains like influenza virus A/Puerto Rico/8/34 for the identification of influenza virus CTL epitopes may have been

in favor of the identification of conserved epitopes (61-63, 87, 125, 158, 160, 216, 245, 263), resulting in an underestimation of the degree of variation in CTL epitopes. To address this issue, we assessed the extent of variation in CTL epitopes using virus-specific $CD8^+$ T-cell clones (chapter 6). PBMC of six HLA-typed study subjects between 35 and 55 years of age were stimulated *in vitro* with an influenza virus that was isolated in 1981. We selected study subjects with HLA-haplotypes for which no CTL epitopes had been described previously in order to prevent the identification of known conserved or variable epitopes. A total of 304 virus-specific CTL clones were obtained by limiting dilution assays with *in vitro* expanded PBMC. The 1981-virus specific $CD8^+$ CTL clones were tested for recognition of autologous BLCL infected with a virus that was isolated more recently (influenza virus A/Netherlands/9/2003). In four out of the six study subjects, CTL clones were identified that were unable to recognize the 2003-virus infected cells. A total of eight out of the 304 (2.6%) 1981-virus specific CTL failed to recognize the recent strain of influenza virus. The 2.6% may be an underestimation, since repeated infections in these individuals may have decreased the frequency of CTL specific for the original version of variable epitopes relative to those specific for conserved epitopes that were boosted during repeated infections. Thus, the number of examples of variable CTL epitopes in the influenza A virus nucleoprotein is increasing, which indicates that there is more variation in these CTL epitopes than was thought previously (185). For some of the 1981-virus specific $CD8^+$ CTL clones long-term cultures could be established, and the MHC class I-restriction and the minimal epitope identified. In addition to the accumulation of amino acid substitutions in CTL epitopes, influenza viruses may use alternate strategies to evade T-cell immunity. It has been shown that NS1 alters the maturation and migration of dendritic cells, preventing the efficient activation of T-cells (77).

During the assessment of the extent of variation in influenza A virus CTL epitopes, we also observed $CD4^+$ T-cell clones that recognized a variable epitope. Escape from $CD4^+$ T-cells has been demonstrated in $CD4^+$ TCR-transgenic mice specific for an immunodominant MHC class II-restricted lymphocytic choriomeningitis virus epitope (45) and has been observed in HIV-positive individuals (105) and hepatitis C virus-immunized chimpanzees (203). However, no immune evasion from $CD4^+$ T-cells have been described in influenza virus infection. We identified the $CD4^+$ T-cell epitope as the HLA-DR*1601-restricted HA_{99-113} epitope (PYDVPDYASLRSLVA) with an evasive amino acid substitution at position 106 (A→V). To exclude that the substitution was induced by antibody-mediated selective pressure, we tested the effect of the HA A106V-substitution on antibody recognition. The A106V amino acid substitution did not affect recognition by virus-specific antibodies, indicating that the substitution may be the result of $CD4^+$ T-cell-driven selective pressure.

Since the proportion of HA_{99-113} (106A)-specific cells only constituted 2.0-6.8% of all virus-specific $CD4^+$ T-cells, which was demonstrated using *in vitro* expanded PBMC of four HLA-DR*1601-positive study subjects, no differences in response could be observed

in polyclonal $CD4^+$ T-cell populations against cells infected with virus with or without the epitope. To investigate the evolution of the $HA_{99\text{-}113}$ epitope sequence, we examined the HA amino acid sequence of 2869 influenza A/H3N2 viruses isolated between 1968 and 2006, submitted to the Influenza Sequence Database (158). The number of viruses containing the HA A106V-substitution went from 5 out of 1252 (1968-1999) to 202 out of 296 viruses in 2002, then declined again, and in 2006 106V-sequences could not be found in the Influenza Sequence Database.

As stated above, there must be a trade-off between intrinsic viral fitness and immune fitness, implying that when immune pressure declines, the possible fitter wild type viruses take over the mutant strains. Alternatively, another (stronger) advantage may select for the wild type sequence. This hypothesis is in agreement with the subdominant nature of the $HA_{99\text{-}113}$ -specific $CD4^+$ T-cell response in HLA-DR*1601-positive individuals and the low prevalence of HLA-DR*1601 in the human population (163). When immune pressure is more robust, the selective pressure results in the fixation of the mutation as is seen for antibody-mediated pressure (227) and CTL-mediated pressure (88, 253). The loss of a T-helper cell epitope may affect antibody or CTL responses, or may affect the anti-viral activity of the $CD4^+$ response more directly, since $CD4^+$ T-cells also possess cytolytic activity (30, 64, 115, 217). At this point it is unclear if and how the temporary escape from $CD4^+$ T-cells has contributed to the improved immune fitness of influenza virus (chapter 8).

Selective pressure against T-helper cell epitopes may have increased by the use of inactivated vaccines, which in addition to neutralizing antibodies against HA and NA, also induce T-helper cells. The inactivated vaccine induces CTL responses inefficiently, if at all, since inactivated antigens do not enter the endogenous antigen processing and MHC class I-presentation pathway. In addition, the number of CTL epitopes on the HA may be limited, since we were unable to identify and generate influenza virus H3N2 HA-specific CTL clones (unpublished data). Alternatively, upon influenza virus infection, HA generated peptides may be simply outnumbered by those liberated from cytoplasmatic proteins NP and M1, which are present in abundance, limiting the induction of HA-specific CTL. Current vaccine design aims at the induction of neutralizing antibodies against HA and NA, which prevent binding to target cells or block the spread of virus, respectively. However, in evaluating the efficacy of novel generation influenza virus vaccines, it would be advisable to consider other correlates of protection than neutralizing antibody titers against HA and NA alone. In the light of antibody-mediated drift and the threat of potential pandemic strains of novel subtypes, the ideal vaccine would provide more universal protective immunity to a variety of homosubtypic and heterosubtypic influenza virus strains (129). For this reason, several developments also aim at the induction of CTL responses (42, 72, 120, 122). However, the efficacy of these vaccines may be affected by variation in CTL epitopes.

Collectively, the data presented in this thesis show that in addition to the selective pressure mediated by humoral host immunity, also CTL mediate a selective pressure on influenza A viruses. In contrast to HIV and SIV, in which the selective pressure is mediated by CTL during the chronic infection of individual hosts, selective pressure on influenza A viruses by CTL immunity takes place at the population level (167). It was found that influenza virus CTL epitopes are either conserved, display variation at non-anchor residues or lose their anchor residues at the cost of viral fitness, which is functionally compensated by the accumulation of co-mutations. In addition, the loss of, or the variation in individual epitopes can affect the influenza A virus-specific CTL response significantly. It not only results in a reduction of the number of virus-specific $CD8^+$ T-cells that can respond to produce cytokines, like IFN-γ, but it also impaired the lytic activity towards infected cells. This in turn may result in delayed clearance of influenza virus infection accompanied with more severe disease and increased mortality. If immune evading influenza viruses result in longer infectious periods in individuals expressing the corresponding HLA molecules, the advantage may lead to fixation of the substitution, also if the HLA molecule is only present in a small proportion of the human population (88). In addition, the loss of $CD4^+$ T-cell reactivity may be expected to contribute to impairment of humoral and cellular immune responses, which is in favor of prolonged viral shedding. Based on these data, we conclude that the high mutation rate and adaptive nature of influenza viruses allows them to evade both humoral and cell-mediated host immunity, which may contribute to their persistence in the human population, and may have implications for the development of vaccines which aim at the induction of virus-specific T-cell responses.

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Nederlandse samenvatting

Influenza virussen veroorzaken acute luchtweginfecties, die in de volksmond griep worden genoemd. Deze infecties zijn zeer besmettelijk. Ieder jaar raken 0,5 tot 1,5 miljoen mensen in Nederland geïnfecteerd met influenza virus, wat wordt aangeduid als een griepepidemie. Een aantal mensen loopt een hoger risico op complicaties na infectie met influenza virus, waaronder de ouderen. Jaarlijks eist de griep in Nederland gemiddeld 1000 dodelijke slachtoffers. Griepepidemieën kunnen mede ontstaan doordat de virale eiwitten, die door neutraliserende antilichamen worden herkend (het hemagglutinine en het neuraminidase), langzaam veranderen. Hierdoor kunnen neutraliserende antilichamen opgewekt door een eerdere infectie met influenza virus of door een eerdere griepvaccinatie de meer recente influenza virussen niet meer herkennen en worden daardoor onwerkzaam. Naast de productie van antilichamen door B-cellen, ook wel humorale immuniteit genoemd, bestaat er ook cellulaire immuniteit, welke gevormd wordt door een bepaald type witte bloedcellen, de zogenaamde T-lymphocyten. De virusspecifieke cytotoxische T-lymphocyten kunnen geïnfecteerde cellen herkennen en opruimen en daarmee de productie van nieuw virus beperken. De herkenning vindt plaats op basis van kleine stukjes viraal eiwit (epitopen), die door de geïnfecteerde cel aan de T-lymphocyten worden gepresenteerd door middel van humaan leukocyten antigeen (HLA). Recentelijk is gebleken dat influenza A virussen ook kunnen ontsnappen aan herkenning door cytotoxische T-lymphocyten door het optreden van mutaties in epitopen. Hierdoor blijft de eliminatie van geïnfecteerde cellen door T-lymphocyten specifiek voor deze veranderde epitopen uit. Het doel van het onderzoek beschreven in dit proefschrift was de mate van variatie in epitopen vast te stellen alsmede het effect van mutaties op de humane influenza virus specifieke cytotoxische T-lymphocyten respons.

In hoofdstuk 2 werd vastgesteld hoe belangrijk een mutatie in een enkel epitoop en het verlies van herkenning door specifieke cytotoxische T-lymphocyten is voor de virusspecifieke immuunrespons. Dit werd onderzocht aan de hand van een natuurlijke mutatie op positie 384 van een HLA-B*2705-gerestricteerd epitoop ($NP_{383-391}$) binnen het influenza virus nucleoproteïne. Het aminozuur op positie 384 van het nucleoproteïne is verantwoordelijk voor de verankering van het $NP_{383-391}$ epitoop in HLA-B*2705 en de waargenomen mutatie voorkomt de binding van het $NP_{383-391}$ epitoop. De cytotoxische T-lymphocytenrespons tegen influenza virussen met de mutatie was significant lager (30%), dan tegen het virus zonder mutatie, wat duidt op het belang van het ontsnappen van influenza virussen voor de cellulaire immuunrespons. Voor dit onderzoek werden met behulp van moleculaire technieken identieke virussen gemaakt die slechts verschillend waren met betrekking tot positie 384 in het nucleoproteïne. De mutatie verantwoordelijk voor de ontsnapping aan herkenning door cytotoxische T-lymphocyten bleek funest voor de replicatie van het virus. In hoofdstuk 3 en 4 wordt beschreven hoe influenza virus niet

altijd kan ontsnappen aan immunologische druk en dat soms co-mutaties noodzakelijk zijn om functionele defecten veroorzaakt door mutaties in epitopen te compenseren. Functionele beperkingen van het virus zouden variatie in epitopen kunnen limiteren en een verklaring zijn voor het geconserveerde karakter van de meeste epitopen. Om dit aan te tonen werden diverse mutaties aangebracht in een geconserveerd dominant HLA-A*0201-gerestricteerd epitoop uit het matrix eiwit ($M1_{58-66}$) en andere geconserveerde epitopen. Vervolgens werd nagegaan of deze mutaties invloed hadden op de replicatie van de virussen *in vitro* en of virusspecifieke cytotoxische T-lymphocyten deze in het laboratorium gemuteerde virussen nog konden herkennen. In de meeste gevallen hadden de mutaties een negatief effect op de replicatie van het virus. Wanneer het virus nog wel repliceerde, werd het nog goed herkend door virusspecifieke cytotoxische T-lymphocyten. Dit zou een verklaring kunnen zijn waarom voor sommige epitopen geen varianten worden waargenomen (hoofdstuk 5).

Slechts enkele influenza virus epitopen vertonen variatie die herkenning door cytotoxische T-lymphocyten kan verhinderen. In hoofdstuk 6 werd de mate van variatie in influenza virus epitopen onderzocht met behulp van klonale cytotoxische T-lymphocyten. Van alle verkregen cytotoxische T-lymphocyten herkende 2,6% een variabel epitoop gepresenteerd door sterk uiteenlopende typen HLA. Gedurende dit onderzoek vonden wij ook variatie in een epitoop dat werd herkend door T-helper lymphocyten. T-helper lymphocyten hebben een ondersteunende en dirigerende functie binnen het immuunsysteem. Dit is de eerste keer dat variatie in een epitoop herkend door T-helper lymphocyten wordt beschreven voor influenza virus. In hoofdstuk 8 wordt beschreven hoe het epitoop werd gelokaliseerd in het virale oppervlakte eiwit hemagglutinine en wat het effect was van de waargenomen mutatie op T-lymphocyten herkenning. Bovendien werd uitgesloten dat de mutatie een gevolg was van het ontwijken van herkenning door antistoffen.

In hoofdstuk 7 wordt nogmaals het belang van een mutatie in een enkel epitoop en het verlies van herkenning door specifieke cytotoxische T-lymphocyten voor de gehele virusspecifieke immuunrespons beschreven. Het betrof hier niet een positie belangrijk voor de verankering van het epitoop in het HLA, maar posities die belangrijk zijn voor herkenning van het epitoop door de T-cell receptor op specifieke T-lymphocyten. Ditmaal werd gebruik gemaakt van een nieuwe techniek, die lytische activiteit van cytotoxische T-lymphocyten kan visualiseren om te laten zien dat de mutatie ook een effect had op de lytische activiteit van de virusspecifieke cytotoxische T-lymphocytenrespons.

Op basis van de data beschreven in dit proefschrift werd geconcludeerd dat het verlies van een enkel epitoop de virusspecifieke immuunrespons significant beïnvloedt. De afname van cellulaire immuniteit tegen influenza virussen kan leiden tot langere infectie en virusuitscheiding, en mogelijk een toename in ziekte en sterfte.

Dankwoord

Graag wil ik iedereen bedanken die betrokken is geweest bij de totstandkoming van dit proefschrift. Op de eerste plaats bedank ik Ab Osterhaus en Guus Rimmelzwaan voor de gelegenheid om promotieonderzoek te verrichten binnen de afdeling Virologie, voor de begeleiding gedurende het onderzoek en de veelvuldige correcties van manuscripten. Graag wil ik Jacco Boon bedanken voor de aanwijzingen op het lab. Tiny Geelhoed-Mieras en Nella Nieuwkoop wil ik bedanken voor hun onmisbare praktische ondersteuning. Yvette Kruyt en Bert Tomson van Sanquin Bloedbank wil ik bedanken voor het selecteren van specifieke bloeddonoren, en de bloeddonoren zelf wil ik bedanken voor hun medewerking aan het onderzoek. Ron Fouchier, Emmie de Wit, Theo Bestebroer en Monique Spronken wil ik bedanken voor het gebruik van hun expertise en middelen met betrekking tot het maken van recombinant influenza virussen. Ook wil ik mijn studenten Jori Symons, Esther Mooiman en Marcel Jonges, en alle overige collega's bedanken voor hun bijdrage. Tot slot bedank ik mijn ouders Jo en Anneke Berkhoff-Niemantsverdriet, mijn broertje Martijn (hem bedank ik ook voor het verzorgen van de opmaak van dit proefschrift), mijn zusjes Judith en Vijitha, m'n vriendinnen Kelly Dalu-Karlas, Gael Heijbers (haar bedank ik ook voor het verzorgen van het promotiefeest), Rini Paradja, Ida Bastiaansen-Rompelman en Claudia Erwich-Faaij, en bovenal m'n bijna echtgenoot Bart Walraven voor hun geweldige steun tijdens mijn promotie.

Curriculum Vitae

Eufemia Gabriella Maria Berkhoff werd geboren op 05 december 1979 te Delft. In 1998 behaalde zij haar Gymnasiumdiploma aan het Sint Stanislas College te Delft. In hetzelfde jaar begon zij aan de studie Biomedische Wetenschappen aan de Faculteit Geneeskunde van de Universiteit Leiden. In het derde studiejaar deed zij onderzoek naar de inductie van immunologische tolerantie na xenotransplantatie bij het Biomedical Primate Research Centre te Rijswijk. In april 2001 verruilde zij deze onderzoeksplek voor een studie naar neurologische afwijkingen ten gevolge van een Schistosoma infectie in het Queen Elizabeth Central Hospital te Blantyre, Malawi. Als afstudeerstage deed zij onderzoek naar de constructie en karakterisatie van recombinant gele koorts virussen voor de expressie van hepatitis C virus antigenen aan het Leids Universitair Medisch Centrum te Leiden. In augustus 2002 behaalde zij het doctoraalexamen. Sinds september 2002 is zij als assistent in opleiding (AIO) werkzaam bij de afdeling Virologie van het Erasmus Medisch Centrum te Rotterdam. Onder leiding van Prof.dr. A.D.M.E. Osterhaus en Dr. G.F. Rimmelzwaan deed zij onderzoek naar het ontsnappen van influenza A virussen aan humane T-cel immuniteit wat heeft geleid tot de totstandkoming van dit proefschrift.

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