Cytotoxic T lymphocyte-mediated immunity to influenza

Cytotoxische T cel gemedieerde immuniteit tegen influenza

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof.dr.ir. J.H. van Bemmel

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 17 september 2003 om 15:45 uur

door

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geboren te Venhuizen

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ISBN number: 90-6734-375-7

The studies described in this thesis were conducted at the Institute of Virology of the ErasmusMC in Rotterdam, the Netherlands, with financial support from Numico Research BV together with the Ministry of Economic Affairs (Center project BTS98081), Novaflu (EU grant QLRT-2001-01034) and SRVI.

Printing of this thesis was financially supported by: Solvay Pharmaceuticals BV, ViroNovative BV, Viroclinics BV, Numico Research BV

Production: Optima Grafische Communicatie, Rotterdam, the Netherlands

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Abbreviations

A	alanine
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
APC	allophycocyanin
BLCL	B lymphoblastic cell-line
C	cysteine
C1R	B cell negative for HLA-A and –B expression
CD	cluster of differentiation
CLIP	class II MHC–associated invariant chain peptide
CMV	cytomegalovirus
cpm	counts per minute
CTL	cytotoxic T lymphocyte
CTLp	precursor frequency of cytotoxic T lymphocyte
D	aspartic acid
DC	dendritic cell
DMSO	di-methyl sulfoxide
E	glutamin
EC_{50}	epitope concentration 50%
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ELIspot	enzyme-linked immunospot
ER	endoplasmatic reticulum
ERAAP	ER aminopeptidase associated with antigen processing
E:T ratio	effector-to-target cell ratio
F	phenylalanine
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HA	hemagglutinin
HAI	hemagglutination inhibition titer
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high pressure liquid chromatography
HRPO	horseradish peroxidase
I	isoleucine
IC_{50}	inhibiting concentration 50%
ICS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IL	interleukin
K	leucine
kb	kilo base pairs

L	lysine
LCMV	lymphocytic choriomeningitis virus
LDA	limiting dilution assay
LST	lymphocyte stimulation test
LPS	lipopolysacharide
M	methionine
M1 and M2	matrix 1 and 2 protein
MAb	monoclonal antibody
MCP	monocyte chemoattractant protein
MDCK	Madin-Darby-Canine-Kidney
MDS	multi-dimensional scaling
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIIC	MHC class II containing compartment
MIP	macrophage inhibitory protein
MOI	multiplicity of infection
MVA	modified vaccinia virus Ankara
NA	neuraminidase
NAC	N-acetyl-L-cysteine
NK	natural killer
NP	nucleoprotein
NS1	non-structural protein
P	proline
PB1/PB2/PA	polymerase proteins
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohemagglutinin
PMA	phorbol 12-myristate 13-acetate
PKR	RNA-dependent protein kinase
R	arginine
RANTES	regulated on activation, normal T cell expressed and secreted
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPE	R-phycoerythrin
rpm	rounds per minute
RSV	respiratory syncitial virus
RPMI	culture medium
S	serine
SD	standard deviation
SEM	standard error of the mean
SIV	simian immunodeficiency virus
SRVI	stichting respiratoire virusinfecties
T	threonine
TAP	transporter associated with antigen processing

$TCID_{50}$	tissue culture-infectious dose 50%
TCR	T cell receptor
Th	T helper lymphocyte
TNF	tumor necrosis factor
V	valine

Chapter 1

General Introduction

History and classification of influenza virus

Influenza viruses are the causative agents of acute respiratory disease, generally termed "flu". The earliest reports of an influenza-like illness include an outbreak in 412BC recorded by Hypocrates (60) and an epidemic in AD1173 (69). The first influenza A virus was isolated in 1933 by Sir Andrewes and Sir Laidlaw (135), quickly followed by influenza B and C virus in 1940 (46) and 1951 respectively (145). Influenza viruses are classified as members of the family of *Orthomyxoviridae*, and are divided into the genus of influenza A and B virus and that of influenza C virus. Influenza A viruses are further divided into subtypes based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). At present 15 different subtypes of HA and 9 subtypes of NA genes are known.

Virus structure and replication

Influenza viruses are enveloped negative stranded RNA viruses with a size of 100-120 nM. The genome of influenza A virus is 13kb in length and consists of eight gene-segments of different sizes. These gene-segments encode nine structural and two non-structural proteins (71, 112) (Tab. 1). Each virus particle contains eight

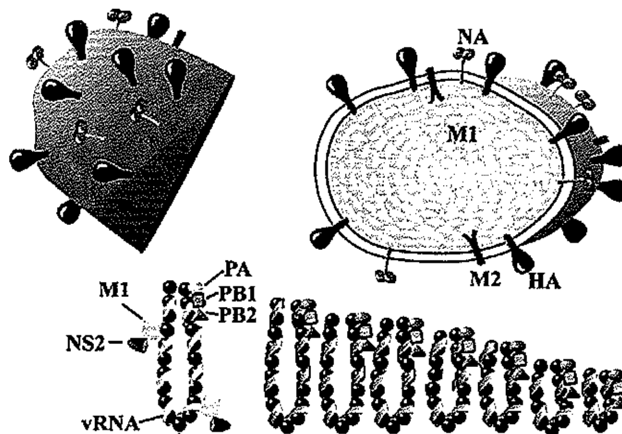

Figure 1: The structure of influenza A virus.
The two glycoprotein spikes HA and NA, together with the M2 protein, are imbedded into the lipid membrane of the virus. Eight gene-segments encoding the 11 different viral proteins are depicted. Each segment binds to a single copy of PA, PB1 and PB2 (polymerase proteins) and multiple copies of the NP protein, forming a RNP complex. M1 and NS2 are associated with the RNP complex and virus membrane. Reproduced with permission from [71].

different gene-segments, each bound to a single copy of the three polymerase proteins (PB1, PB2 and PA) and multiple copies of the nucleoprotein (NP). This RNP complex is surrounded by a matrix of protein (M1 and NS2) and a lipid membrane, which is taken from the host cell. Inserted into the lipid membrane are

Table 1: Influenza A virus segments and encoded proteins

RNA segment (nucleotides)	Gene product (amino acids)	Molecules per virion
1. (2341)	Polymerase PB2 (759)	30-60
2. (2341)	Polymerase PB1 (757)	30-60
	PB1-ORF2 (87)	
3. (2233)	Polymerase PA (716)	30-60
4. (1778)	Hemagglutinin (566)	500
5. (1565)	Nucleoprotein (498)	1000
6. (1413)	Neuraminidase (454)	100
7. (1027)	Matrix protein M1 (252)	3000
	Matrix protein M2 (97)	20-60
8. (890)	Non structural protein NS1 (230)	-
	Non structural protein NS2 (121)	130-200

three different proteins, the M2 protein, HA and NA protein (71, 112) (figure 1).

Upon attachment of the virus to sialic acids on the surface of airway epithelial cells, the virus can enter the cells by receptor-mediated endocytosis via chlatrin-coated pits. Fusion of the virus membrane with the endosomal membrane allows the RNP complex to be released into the cytoplasm. Prior to membrane fusion, the HA molecule, responsible for binding to the sialic acid residues, must be cleaved into a HA1 and HA2 subunit by serine proteases, which allows a conformational change necessary for fusion. The conformational change is triggered by a low pH in the

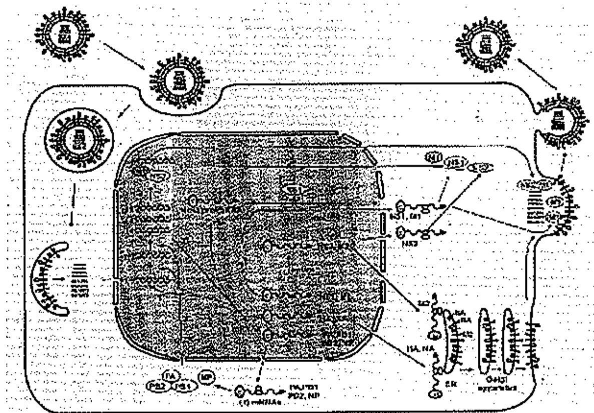

Figure 2: Replication cycle of influenza viruses. From Robert A. Lamb and Robert M. Krug's "Orthomyxoviridae: The viruses and their replication" in Fields Virology, 4th edition, Lippincott Williams & Wilkins. 2001. p 1505

endosomes, which also induces the dissociation of the RNP complexes from M1 proteins. The individual RNP complexes are than transported from the cytoplasm of the infected cell into the nucleus, where transcription and translation can commence. First, the viral RNA (vRNA) molecules are transcribed into mRNA molecules and subsequently translated into proteins. During the first few hours after infection mainly NP and NS1 proteins are produced. The function of the NS1 protein is to counteract innate immune defense mechanisms and inhibit host mRNA transport, while the NP protects the vRNA and induces a shift from mRNA production to vRNA production in the nucleus. This shift in RNA production is as early as 3 hours after infection and allows the production of new progeny virus particles. At this later stage in the infection process more HA, NA and M1 proteins are produced, which are necessary to form viral particles. These molecules together with newly formed RNP complexes are transported to the membrane of the infected cell where new virus particles are formed (71, 112) (figure 2).

Pathogenesis of influenza A virus in humans

Infection with influenza viruses can take place after inhalation of respiratory secretions from infected individuals (2, 105). These aerosols are deposited in the respiratory tract, initiating infection in the ciliated columnar epithelial cells (67, 154) of the tracheobronchial epithelium, from where it may spread throughout the entire respiratory tract (86, 87). Viral shedding can be detected as early as 24 hours after infection, peaks around 48 hours, and declines to low levels at day 5-6. The amount of virus-shedding is related to the severity of illness and temperature elevation (112).

Aerosol formation, ideal for depositing viral particles in the respiratory tract. From www.health.state.mn.us/ divs/dpc/adps/flu2/influenza.htm.

The incubation period for influenza is 2-3 days, but can be as short as 24 hours after infection. Good indicators of an infection with influenza virus are a sudden onset of illness, cough, malaise and feverishness, while diarrhea, dizziness and chest pain are among the symptoms not associated with influenza virus (114). The clinical symptoms associated with an influenza virus infection depend on the immune status, patient age as well as the type of influenza virus. Severe complications include acute bronchitis, laryngotracheobronchitis (croup), bronchilitis, bronchopneumonia with multiple organ dysfunction and bacterial pneumonia (114).
In immune-competent individuals influenza virus infection during the winter epidemics rarely leads to severe complications and rarely results in death. However in immune-compromised patients, in particular the elderly, infections with influenza

viruses can lead to severe disease and death. In the Netherlands alone, 1000-4000 excess deaths in the elderly population are attributed to influenza virus infection each year (124). Besides the elderly, diabetes patients, patients suffering from respiratory conditions (asthmatics, chronic obstructive pulmonary disease patients and cystic fibrosis) and the immune-compromised patients (transplant recipients and HIV-infected individuals), also belong to the risk-group associated with influenza virus infection.

Host range of influenza viruses

Influenza A viruses have been known to infect many different animal species including birds (mainly ducks and waterfowl), horses, whales, seals, humans and pigs (figure 3) (39, 71). Aquatic birds or waterfowl are believed to be the main reservoir for influenza A virus. From this reservoir, direct or indirect transmissions via an intermediate host, allow the virus to cross species barriers. Examples of direct transmission from birds to pigs, domestic poultry, horses and minks are abundant (reviewed in (71, 112)), while direct transmissions from aquatic birds to humans are rare (81). The latter was explained by the inefficient replication of avian influenza viruses in man (5).
Several transmissions of avian influenza A viruses from domesticated birds, like turkeys and chickens, to humans have been described. In 1997, an avian influenza A virus of the H5N1 subtype, circulating in chickens in the bird markets of Hong Kong, infected 18 individuals, of whom six died (29, 139). A similar event occurred in 2003, when one person died of a confirmed infection with an avian H5N1 virus.

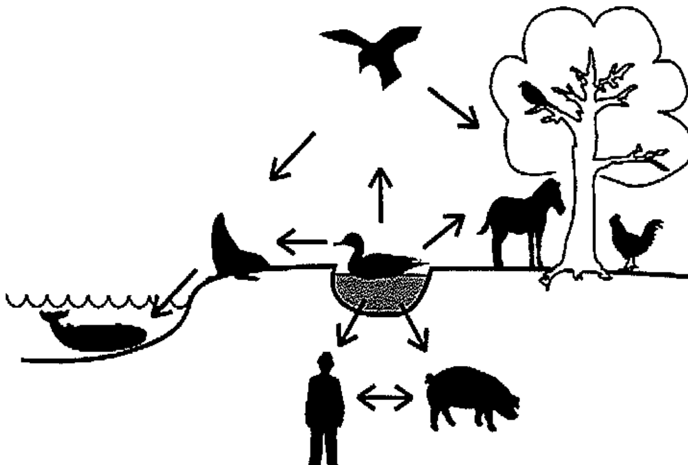

Figure 3: Host range of influenza A viruses.
Epidemiological studies have shown that waterfowl are the main reservoir for influenza A viruses, from which transmission can occur to other species. Reproduced with permission from [71].

In addition to these transmissions with H5N1 viruses, two cases were reported to be infected with a H9N2 virus of avian origin in 1999 in the southern part of China (62, 63). Finally, many transmissions of an H7N7 avian influenza virus have been recorded in the Netherlands in 2003 during an outbreak of fowl plague. These transmissions occurred mainly in poultry workers and veterinarians, causing conjunctivitis in a third of the cases (personal communication R.A.M. Fouchier and A.D.M.E. Osterhaus). Besides avian-to-human transmissions, influenza viruses circulating in pigs can also infect humans and cause severe disease (127, 131, 160). The susceptibility of the pig for both avian and human influenza viruses makes it an ideal 'mixing' vessel or intermediate host, capable of generating avian-human reassortant viruses, which can than be transmitted to humans (132, 157). This scenario is believed to have produced both the 1957 and 1968 pandemic influenza A viruses (78, 158).

Epidemiology and pandemics

Influenza A viruses have been around for many centuries and are thought to have caused at least ten pandemics in the last 450 years. They have also, along with other infectious diseases, played a significant role in shaping the demographics of the current world. In the last century three pandemics have swept the globe, Spanish influenza in 1918 being the most devastating and killing between 20 and 40 million people worldwide. The other two pandemics occurred in 1957 (Asian flu) and 1968 (Hong Kong flu) (figure 4). A pandemic is usually preceded by the introduction of a new subtype of influenza A virus in the human population who lack pre-existing immunity against this subtype. Currently, three subtypes of influenza A virus circulate in the human population. The H3N2 subtype, introduced in 1968, is still circulating together with viruses of the H1N1 subtype, which was reintroduced in 1977. Finally in 2001 a new subtype (H1N2) emerged, which is a reassortant virus of the human H1N1 and H3N2 viruses (57, 163).

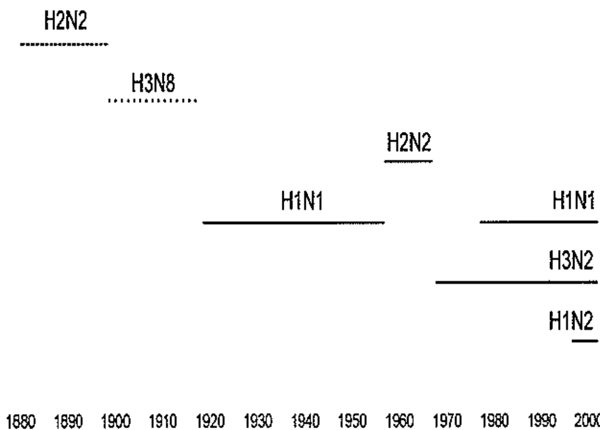

Figure 4: Subtypes of influenza A viruses circulating in humans in the past 125 years. The first H2N2 (-----) and the H3N8 epidemic (····) have been determined by serology.

Influenza A viruses escape pre-existing immunity in humans by means of antigenic drift and antigenic shift. Antigenic drift is a process involving the accumulation of point mutations in the HA and

NA gene as a result of antibody-mediated immune pressure. These mutations allow the virus to escape recognition by neutralizing antibodies induced during a previous infection with influenza virus. Antigenic drift is thought to cause the yearly epidemics with new variants of influenza A virus of the same subtype. A second mechanism of influenza A virus to escape pre-existing immunity is called antigenic shift. This unique ability of segmented RNA viruses can occur after an infection of a single cell (read host) with two subtypes (a human and a non-human strain) of influenza A virus. The gene-segments of these two viruses can be exchanged, resulting in new progeny virus containing RNA segments of both viruses. If the progeny virus or reassortant virus has acquired the HA and/or NA gene of the non-human virus strain, a potential new pandemic virus is produced, capable of replicating efficiently in humans due to the "humanized" internal genes and able to infect many individuals due to the lack of pre-existing antibodies specific for the HA and/or NA surface glycoproteins. Antigenic shift is thought to have occurred in 1957 and 1968, when the HA and/or NA gene of an avian influenza A virus were incorporated into a human strain of influenza virus.

Immunity to influenza virus

As a model pathogen, influenza viruses have been used to study many different aspects of the immune system. Especially in mice, although not the natural host for influenza viruses, the importance of CD8+-, CD4+ T cells, innate immunity, antibody producing B cells, cytokines and the complement system in protective immunity against viruses has been identified.
These investigations have increased the knowledge of the immune system and the interplay between the immune system and viruses considerably. Unfortunately, and not without reason, less is known about the immunity and especially the cell-mediated immunity to influenza virus in humans. A difficulty in studying cellular immunity to influenza viruses in humans is the high degree of diversity among individuals with regards to genetic background, history of influenza virus infections and age.

Innate immunity

The innate immune response or non-specific immune response is the first line of defense and is detectable within 24 hours after experimental infection with influenza virus (112). Innate immunity is mediated by a cellular arm and the production of antiviral proteins.
Upon infection with influenza viruses, epithelial cells and monocytes/macrophages start producing chemokines and cytokines. Epithelial cells produce RANTES, MCP-1 and IL-8 (1, 91), while macrophages produce IFN-α/ß, RANTES, MIP-1α, MIP-1ß, MCP-1, MCP-3, MIP-3α and IP-10 (20, 76, 90, 130, 137). The production of these chemokines result in the recruitment of immune cells to the site of infection (20, 137). These cells, including macrophages, neutrophils and natural killer (NK) will lead to the production of more cytokines, chemokines and other anti-viral

proteins. Furthermore, cellular debris and infected cells are phagocytosed, while NK cells kill virus-infected cells following activation by the HA protein of influenza virus (89). Type I interferons (IFN-α/ß), produced by monocytes/macrophages, are very important in controlling influenza virus infections as was shown in STAT-1 knockout mice (48) and in humans (68, 108, 125). IFN-α/ß induce an antiviral state in neighboring cells due to the expression of PKR, RNAaseL/2'-5' oligosynthetase and Mx proteins. Besides IFN-α/ß, double-stranded RNA initiates PKR activation, resulting in phosphorylation of eukaryotic initiation factor 2α. This factor reduces translation initiation and hence the synthesis of cellular proteins. In addition to activation of non-specific antiviral immunity, IFN-α/ß is involved in the recruitment of monocytes/macrophages and T cells. It also enhances antigen presentation via increased major histocompatibility complex (MHC) expression and maturation of antigen presenting cells (APC).
Another component of the innate immune system, C3, was also shown to link the innate and adaptive immunity (80). The C3 factor, part of the complement system and essential in chemotaxis and the formation of pore complexes, provides an essential signal for CD4+ T cells. Besides C3, C2R double negative mice also showed delayed T cell responses to influenza virus.

Adaptive immunity

In response to infection with influenza virus also adaptive immunity is induced, mediated by B and T cells. The T- and B cell responses are initiated by professional APC in the lymph nodes. The APC can stimulate both CD4+ (T helper or Th cell) and CD8+ T cells (CTL). The activated Th-cells assist in the production of antibodies by B cells and in the induction of a vigorous CTL response.
APC, like dendritic cells (DC), macrophages and B cells, are capable of expressing both human leukocyte antigen (HLA) class I and II molecules. Th cells recognize peptides (10-30 amino acids long), derived from an exogenous protein source by endocytosis, in the groove of HLA class II molecules (19). In the endocytic compartment peptides are generated after enzymatic cleavage of viral proteins (152). Following fusion of the endosome with a second vesicle, forming the HLA class II containing compartment (MIIC), the CLIP fragment is exchanged for the peptide with the aid of the HLA-DM chain (113). Next the peptide/HLA class II complex is transported to the surface of the APC.
At the same time, APC, like most cells of the body, present peptides (8-12 amino acids long), in the context of HLA class I molecules (61, 164). These peptides are derived from newly synthesized proteins in the cytosol (e.g. self of viral) and cleaved by the proteosome into small peptide fragments. These fragments are then transported into the endoplasmatic reticulum (ER) by TAP (transporter associated with antigen processing), where they can bind to and stabilize HLA class I molecules. In the ER additional N-terminal cleavage by the ERAAP (ER aminopeptidase associated with antigen processing) is possible (134), increasing the stability of the HLA-peptide complex. Ultimately these complexes are transported through the Golgi-apparatus to the surface of the cell. DC, but also macrophages,

have been known for their ability to present peptides in the context of HLA class I derived from exogenous protein, which is known as cross-priming (12, 129).

B cell immunity

The antibody response plays an important role in controlling the virus after a primary infection with influenza virus (106, 146). In mice, antibody-secreting cells become detectable in the lymph node between days 4-5 after primary infection (66, 77, 143), and in the respiratory tract between days 5-7 (66, 143). These responses peak at day 5-7 and between day 10-20 respectively. The mucosal antibody production following a primary infection is dominated by IgM antibodies, which are replaced by IgA and IgG at later stages in the response (66, 77, 109, 111). The IgA response can often still be detected up to 3-5 months after infection (109) and provide protection against a reinfection. During a secondary response mainly IgA antibodies are secreted, although IgG-type antibodies, transudated from the serum, are present.
The target proteins for influenza virus-specific antibodies are the HA, NA, NP and M proteins (118). Antibodies specific for the HA and NA can provide protection from influenza virus infection, since these antibodies interfere with attachment (HA) and release of progeny virus (NA) (30, 42, 110).
In humans, the antibody response in serum is long lasting and has been shown to be significant for 5-7 years (32). In addition, people exposed to viruses of the H1N1 subtype before 1957 were less susceptible to infection with H1N1 virus after its reintroduction in 1977. The level of protection in humans correlates with the level of HA- and NA-specific antibodies against the homologous strain, confirming the importance of virus-specific antibodies (30, 32). Virus-neutralizing antibodies are also responsible for selective pressure resulting in antigenic drift allowing the virus to escape from these antibodies. Originally reported for antibody responses following infection with influenza virus, original antigenic sin describes a phenomenon in which the antibody response elicited in an individual after a secondary viral infection reacts more strongly to the viral variant that originally infected the individual (43, 156).

T cell immunity

From studies using "knockout" mice, the importance of T cells during an influenza virus infection was recognized (8, 50, 162), since T cell depleted mice are unable to resolve the infection and eventually die. CD4+ T cells kill virus-infected cells and are crucial for the induction of an antibody response. The contribution of CD8+ T cells to the influenza virus-specific immune response is controversial. Several studies have investigated the role of these cells in the protection from subsequent influenza virus infections. Despite the existence of heterosubtypic cross-reactive CTL, CTL provide little or no protection against reinfection with a different subtype of influenza virus (for review (128)). However, CD8+ T cells have been shown to control viral replication and shorten the duration of viral shedding (45). This was

demonstrated in immune-competent mice following passive transfer of virus-specific CD8+ T cells (144). Furthermore CD8+ T cell-depleted mice shed virus for prolonged periods of time. In humans, the significance of CD8+ T cells was demonstrated in a study by McMicheal, who showed that the pre-challenge levels of

Table 2: Known influenza A virus CTL epitopes

Restriction element (HLA-)	Protein (amino acids)	Amino acid sequence	Reference
A1	PB1 (591-599)	VSDGGPNLY	(35)
	NP (44-52)	CTELKLSDY	(35)
A*0201	M1 (58-66)	GILGFVFTL	(6, 56)
	M1 (59-68)	ILGFVFTLTV	(56)
	NS1 (122-130)	AIMDKNIIL	(88)
	NS1 (123-132)	IMDKNIILKA	(88)
	NA (213-221)	CVNGSCFTV	(159)
A3	NP (265-273)	ILRGSVAHK	(35)
	M1 (27-35)	RLEDVFAGK	(149)
A*6801	NP (91-99)	KTGGPIYKR	(64)
B8	NP (380-388)	ELRSRYWAI	(140)
B*1402	NP (146-154)	TTYQRTRAL	(33)
B*2702	NP (381-388)	LRSRYWAI	(150)
B*2705	NP (383-391)	SRYWAIRTR	(72)
	NP (184-194)	RRSGAAVAAHK	(75)
B*35	M1 (128-135)	ASCMGLIY	(38)
B*3701	NP (339-347)	EDLRVLSFI	(148)
B44	NS1 (158-166)	GEISPLPSL	(34)
	NP (338-346)	FEDLRVLSF	(34)
	NP (379-387)	LELRSRYWA	(34)
	M2 (7-15)	VETPIRNEW	(74)
Cw*0102	M1 (51-59)	ILSPLTKGI	(3)
	M1 (51-60)	ILSPLTKGIL	(3)
	M2 (85-94)	AVDADDSHFV	(3)
	M2 (85-94)	DADDSHFVSI	(3)
	NP (470-479)	KATSPIVPSL	(3)

Partly taken from the influenza virus sequence database (www.flu.lanl.gov).

virus-specific CTL was associated with accelerated clearance of the virus from the respiratory tract (98). Finally, the immune pressure on two CTL epitopes ($NP_{380-388}$ and $NP_{383-391}$), believed to be associated with escape from pre-existing CTL immunity, also indicates an important role for CTL-mediated immune pressure (126, 153) during an influenza virus infection.

The dynamics of the CD4+ and CD8+ cellular immune response is similar. In the BAL and MLN of mice, tetramer staining showed that virus-specific CD8+ T cells

were detectable on day 5 after primary infection and on day 3-4 after a secondary infection (36). Virus-specific CTL remain present for prolonged periods of time could potentially provide protection against infection with a different subtype of influenza A virus (70). In humans, influenza virus-specific T cell responses are detectable between day 3 and 6 after infection and return to baseline levels by day 28 (37). CTL specific for influenza virus appear in the blood at day 6-14 of infected or vaccinated individuals and disappear by day 21 (41).
From the early 1980's it was known that influenza virus-specific CTL recognize most human influenza A virus subtypes (13, 17, 97). Later on it was discovered that many CTL epitopes are generated from the internal proteins of the virus (NP, M1, PB1, Tab. 2). Since these proteins display a high degree of sequence homology in the different subtypes of influenza A virus (44, 96, 99, 166), CTL are able to recognize various subtypes of influenza A virus (heterosubtypic recognition). Homosubtypic CTL, capable of recognizing a single subtype of influenza A virus, are usually specific for the glycoproteins HA and NA (75, 159).
During the early studies on CTL responses in mice, it was found that virus-specific CTL responses were directed to a limited number of epitopes, a phenomenon now called immunodominance (11, 165). The underlying principle of immunodominance is an area of intense research and many factors have been shown to be involved. The most dominant factors influencing immunodominance are thought to be epitope abundance and epitope affinity, although several studies have demonstrated that high affinity epitopes are not always the immunodominant ones (26, 107). Other factors influencing immunodominance hierarchies are; IFN-γ expression (4), available TCR repertoire (7), history of infections (18), viral persistence (161), HLA background (7, 147, 150), the nature of the immunogen (28), antigen processing (27, 104) and competition (147, 150).

Aging and the immune system

Aging is associated with a decrease in immune function, resulting in a higher prevalence of infectious diseases like pneumonia and urinary tract infections in the elderly. Although the decline in immune functioning is the primary cause of this, underlying mechanisms, like diseases and nutritional status, play a role. The role of the immune system in aging is apparent from the association between longevity and HLA-polymorphism (73, 122, 141) and the well functioning immune system of centenarians (31). The effects of nutrition and diseases on the functioning of immune system lead to the installation of the so-called "SENIEUR" protocol. This protocol is used for studies involving aging and excludes confounding effects due to nutrition and disease (85).
The decline in immune function is called immunosenescence and entails both the humoral and cellular arms of the immune system as well as the aging environment and hormone status of the aging individual. Although the immunological changes are very diverse and occur in most cells of the immune system, the number of immune cells is similar in young and elderly individuals (102, 123). The exception is NK cells, which increase significantly in number in the elderly (15, 116, 136). The

reduction in immune responsiveness of immune cells of aging individuals is likely to be caused by functional defects in these cells and generally believed to occur mainly in T cells (102, 103). Among the most striking changes is the shift from CD45RA "naïve" to CD45RO "memory" type of T cells (47, 65, 151), higher number of HLA-DR+ T cells (138), reduction in telomere length (40) and the loss of CD28 expression (16), in particular in the CD8+ T cell population. The CD28 molecule is involved in co-stimulation and could explain the reduced CTL response in the elderly (92, 93, 119, 120). CTL have also been reported to produce less perforin and granzyme, resulting in less efficient killing of virus-infected cells (52). CD4+ T cells were shown to have a reduced signaling capacity (22, 115) which may result in a lower IL-2 production and reduced ability to proliferate (40).
DC function has been reported to remain stable during aging, in fact a few studies suggest an increased ability to stimulated antigen-specific T cells. However, others claim that monocytes and macrophages, a second type of APC, produce less pro-inflammatory cytokines *in vitro* following stimulating with LPS (53, 95), while in humans, alveolar macrophages have a reduced accessory cell function in aging individuals (169).
B cell function is also reduced in the elderly. This is of particular interest since influenza virus vaccinations aim to induce virus-neutralizing antibody responses, especially in the elderly. In aging mice the germinal center formation is delayed 1-2 days and the hypermutation rate in B cells is reduced (168), something that is believed to be caused by a defective T cell compartment in these mice. As a result of the reduced immune responsiveness in the elderly the immune response following natural infection or influenza vaccination is markedly reduced (9, 10, 124).

Bioactive compounds and the immune system

The impact of nutrition on the immune system was first identified in the mid-1980s and since then this relationship has been investigated intensively (23, 24, 58, 59, 82, 84, 117). A positive role for vitamins and trace elements (vitamin A, (133), B6 (101, 142), B9 (14), C (14, 117), E (100), zinc (79), selenium (94)) on immunological parameters have been described, although these data were not always confirmed (49, 54, 55). During the mid-1990s the effects of other bioactive compounds like poly-unsaturated fatty acids, amino acids, phytosterols, isoflavones and several Chinese herbal tea extracts (21, 155, 167) were studied. Again a rather pleiomorphic set of data was generated, claiming positive, negative or no effect on different immunological parameters. The discrepant data are explained to some extent by differences in study-cohort; e.g. age, gender, SENIEUR protocol and social background, as well as differences in the studied immunological parameters; e.g. antigenic stimulus vs. mitogenic stimulus, cell count, proliferative responses, antibody responses and NK activity. Often vaccination against influenza virus is used as a model system to study the effect of micronutrients on the influenza virus-specific immune response (23, 25, 51, 83, 121). It is important to note that differences in the dose of supplemented micronutrients, the duration of the study and the combination of different nutrients influence the outcome of these studies.

Outline of the thesis

Although some aspects of virus-specific CTL immunity are poorly understood, CTL are generally considered to play a pivotal role in the control of viral infections. The aim of the study was to investigate the effect of HLA background, aging and nutrition on virus-specific CTL responses, using influenza as a model. Investigating CTL immunity to influenza virus is facilitated by the large number of study subjects that have been exposed to the virus, the large number of CTL epitopes that have been identified, the susceptibility of PBMC to infection and the availability of a plethora of virus strains and virus-specific CTL clones.
In order to determine the role of HLA background on the influenza virus-specific CTL response, a cohort of HLA-A and HLA-B genotyped donors was established. These donors were divided into three groups of HLA-A and –B identical donors, each group sharing two or three of the four alleles with other groups. In chapter 2, the CTL response specific for individual influenza A virus CTL epitopes was determined in PBMC of the HLA-typed donors *ex vivo.* Immunodominant epitopes were identified and it was shown that the magnitude of the CTL response specific for the virus or individual epitopes was dependent on HLA background. In addition, the extent to which individual HLA-A and –B alleles contribute to virus-specific CTL responses was investigated in chapter 3. A preferred HLA usage in the influenza virus-specific CTL response was observed in addition to functional diversity in CD8+ T cells which was dependent on the HLA allele.
Besides HLA background, the effect of aging on the CTL response was determined. Several parameters were studied, ranging from precursor frequency of influenza virus-specific T cells in PBMC of donors of various ages to proliferative capacity upon stimulation and capacity to lyse virus-infected target cells (chapter 6). It was found that the number of virus-specific CTL in PBMC did not fluctuate during aging. The impaired CTL activity in the elderly was related to functional defects in these cells. A new immunodominant HLA-B*3501-restricted CTL epitope is described in chapter 4. This epitope exhibited extensive amino acid sequence variation over time. This amino acid variation was associated with escape from CTL recognition (chapter 4 and 5). In chapter 5 recognition of variants of the newly identified HLA-B*3501-restricted epitope by CTL was studied in greater detail. It was hypothesized that consecutive infections with variant viruses select for cross-reactive CTL.
Finally, immune-stimulatory properties of bioactive compounds were investigated *in vitro*. Two compounds were identified which enhanced the influenza virus-specific T cell immunity.

References

1. **Adachi, M., S. Matsukura, H. Tokunaga, and F. Kokubu.** 1997. Expression of cytokines on human bronchial epithelial cells induced by influenza virus A. Int Arch Allergy Immunol. **113:**307-11.
2. **Alford, R. H., J. A. Kasel, P. J. Gerone, and V. Knight.** 1966. Human influenza resulting from aerosol inhalation. Proc Soc Exp Biol Med. **122:**800-4.
3. **Andersen, M. H., I. Sondergaard, J. Zeuthen, T. Elliott, and J. S. Haurum.** 1999. An assay for peptide binding to HLA-Cw*0102. Tissue Antigens. **54:**185-90.
4. **Badovinac, V. P., A. R. Tvinnereim, and J. T. Harty.** 2000. Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. Science. **290:**1354-8.
5. **Beare, A. S., and R. G. Webster.** 1991. Replication of avian influenza viruses in humans. Arch Virol. **119:**37-42.
6. **Bednarek, M. A., S. Y. Sauma, M. C. Gammon, G. Porter, S. Tamhankar, A. R. Williamson, and H. J. Zweerink.** 1991. The minimum peptide epitope from the influenza virus matrix protein. Extra and intracellular loading of HLA-A2. J Immunol. **147:**4047-53.
7. **Belz, G. T., P. G. Stevenson, and P. C. Doherty.** 2000. Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. J Immunol. **165:**2404-9.
8. **Bender, B. S., T. Croghan, L. Zhang, and P. A. Small, Jr.** 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J Exp Med. **175:**1143-5.
9. **Bender, B. S., J. B. Ulmer, C. M. DeWitt, R. Cottey, S. F. Taylor, A. M. Ward, A. Friedman, M. A. Liu, and J. J. Donnelly.** 1998. Immunogenicity and efficacy of DNA vaccines encoding influenza A proteins in aged mice. Vaccine. **16:**1748-55.
10. **Bernstein, E., D. Kaye, E. Abrutyn, P. Gross, M. Dorfman, and D. M. Murasko.** 1999. Immune response to influenza vaccination in a large healthy elderly population. Vaccine. **17:**82-94.
11. **Berzofsky, J. A.** 1988. Immunodominance in T lymphocyte recognition. Immunol Lett. **18:**83-92.
12. **Bevan, M. J.** 1995. Antigen presentation to cytotoxic T lymphocytes in vivo. J Exp Med. **182:**639-41.
13. **Biddison, W. E., S. Shaw, and D. L. Nelson.** 1979. Virus specificity of human influenza virus-immune cytotoxic T cells. J Immunol. **122:**660-4.
14. **Bogden, J. D., A. Bendich, F. W. Kemp, K. S. Bruening, J. H. Shurnick, T. Denny, H. Baker, and D. B. Louria.** 1994. Daily micronutrient supplements enhance delayed-hypersensitivity skin test responses in older people. Am J Clin Nutr. **60:**437-47.

15. **Borrego, F., M. C. Alonso, M. D. Galiani, J. Carracedo, R. Ramirez, B. Ostos, J. Pena, and R. Solana.** 1999. NK phenotypic markers and IL2 response in NK cells from elderly people. Exp Gerontol. **34:**253-65.
16. **Boucher, N., T. Dufeu-Duchesne, E. Vicaut, D. Farge, R. B. Effros, and F. Schachter.** 1998. CD28 expression in T cell aging and human longevity. Exp Gerontol. **33:**267-82.
17. **Braciale, T. J., M. E. Andrew, and V. L. Braciale.** 1981. Heterogeneity and specificity of cloned lines of influenza-virus specific cytotoxic T lymphocytes. J Exp Med. **153:**910-23.
18. **Brehm, M. A., A. K. Pinto, K. A. Daniels, J. P. Schneck, R. M. Welsh, and L. K. Selin.** 2002. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. Nat Immunol. **3:**627-34.
19. **Bryant, P. W., A. M. Lennon-Dumenil, E. Fiebiger, C. Lagaudriere-Gesbert, and H. L. Ploegh.** 2002. Proteolysis and antigen presentation by MHC class II molecules. Adv Immunol. **80:**71-114.
20. **Bussfeld, D., A. Kaufmann, R. G. Meyer, D. Gemsa, and H. Sprenger.** 1998. Differential mononuclear leukocyte attracting chemokine production after stimulation with active and inactivated influenza A virus. Cell Immunol. **186:**1-7.
21. **Calder, P. C.** 1998. Dietary fatty acids and lymphocyte functions. Proc Nutr Soc. **57:**487-502.
22. **Chakravarti, B.** 2001. T-cell signaling--effect of age. Exp Gerontol. **37:**33-9.
23. **Chandra, R. K.** 1992. Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. Lancet. **340:**1124-7.
24. **Chandra, R. K.** 1992. Nutrition and immunity in the elderly. Nutr Rev. **50:**367-71.
25. **Chandra, R. K., and S. Puri.** 1985. Nutritional support improves antibody response to influenza virus vaccine in the elderly. Br Med J (Clin Res Ed). **291:**705-6.
26. **Chen, W., L. C. Anton, J. R. Bennink, and J. W. Yewdell.** 2000. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. Immunity. **12:**83-93.
27. **Chen, W., C. C. Norbury, Y. Cho, J. W. Yewdell, and J. R. Bennink.** 2001. Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. J Exp Med. **193:**1319-26.
28. **Cho, Y., S. Basta, W. Chen, J. R. Bennink, and J. W. Yewdell.** 2003. Heat-aggregated noninfectious influenza virus induces a more balanced CD8(+)-T-lymphocyte immunodominance hierarchy than infectious virus. J Virol. **77:**4679-84.
29. **Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. **351:**472-7.

30. **Clements, M. L., R. F. Betts, E. L. Tierney, and B. R. Murphy.** 1986. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol. **24:**157-60.
31. **Cossarizza, A., C. Ortolani, D. Monti, and C. Franceschi.** 1997. Cytometric analysis of immunosenescence. Cytometry. **27:**297-313.
32. **Couch, R. B., and J. A. Kasel.** 1983. Immunity to influenza in man. Annu Rev Microbiol. **37:**529-49.
33. **DiBrino, M., K. C. Parker, D. H. Margulies, J. Shiloach, R. V. Turner, W. E. Biddison, and J. E. Coligan.** 1994. The HLA-B14 peptide binding site can accommodate peptides with different combinations of anchor residues. J Biol Chem. **269:**32426-34.
34. **DiBrino, M., K. C. Parker, D. H. Margulies, J. Shiloach, R. V. Turner, W. E. Biddison, and J. E. Coligan.** 1995. Identification of the peptide binding motif for HLA-B44, one of the most common HLA-B alleles in the Caucasian population. Biochemistry. **34:**10130-8.
35. **DiBrino, M., T. Tsuchida, R. V. Turner, K. C. Parker, J. E. Coligan, and W. E. Biddison.** 1993. HLA-A1 and HLA-A3 T cell epitopes derived from influenza virus proteins predicted from peptide binding motifs. J Immunol. **151:**5930-5.
36. **Doherty, P. C., J. M. Riberdy, and G. T. Belz.** 2000. Quantitative analysis of the CD8+ T-cell response to readily eliminated and persistent viruses. Philos Trans R Soc Lond B Biol Sci. **355:**1093-101.
37. **Dolin, R., B. R. Murphy, and E. A. Caplan.** 1978. Lymphocyte blastogenic responses to influenza virus antigens after influenza infection and vaccination in humans. Infect Immun. **19:**867-74.
38. **Dong, T., D. Boyd, W. Rosenberg, N. Alp, M. Takiguchi, A. McMichael, and S. Rowland-Jones.** 1996. An HLA-B35-restricted epitope modified at an anchor residue results in an antagonist peptide. Eur J Immunol. **26:**335-9.
39. **Easterday, B. C.** 1975. Animal Influenza, p. 449-481. *In* E. D. Kilbourne (ed.), The influenza viruses and influenza. Academic Press, Orlando.
40. **Effros, R. B.** 2001. Immune system in the elderly, p. 324-350. *In* E. J. Masoro, and S. Austad (eds), Handbook of the Biology of Aging (5th edn). Academic Press, London.
41. **Ennis, F. A., A. H. Rook, Y. H. Qi, G. C. Schild, D. Riley, R. Pratt, and C. W. Potter.** 1981. HLA restricted virus-specific cytotoxic T-lymphocyte responses to live and inactivated influenza vaccines. Lancet. **2:**887-91.
42. **Epstein, S. L., J. A. Misplon, C. M. Lawson, E. K. Subbarao, M. Connors, and B. R. Murphy.** 1993. Beta 2-microglobulin-deficient mice can be protected against influenza A infection by vaccination with vaccinia-influenza recombinants expressing hemagglutinin and neuraminidase. J Immunol. **150:**5484-93.
43. **Fazekas de St, G., and R. G. Webster.** 1966. Disquisitions of Original Antigenic Sin. I. Evidence in man. J Exp Med. **124:**331-45.

44. **Fleischer, B., H. Becht, and R. Rott.** 1985. Recognition of viral antigens by human influenza A virus-specific T lymphocyte clones. J Immunol. **135:**2800-4.
45. **Flynn, K. J., G. T. Belz, J. D. Altman, R. Ahmed, D. L. Woodland, and P. C. Doherty.** 1998. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. Immunity. **8:**683-91.
46. **Francis, T.** 1940. A new type of virus from epidemic influenza. Science. **92:**405-406.
47. **Gabriel, H., B. Schmitt, and W. Kindermann.** 1993. Age-related increase of CD45RO+ lymphocytes in physically active adults. Eur J Immunol. **23:**2704-6.
48. **Garcia-Sastre, A., R. K. Durbin, H. Zheng, P. Palese, R. Gertner, D. E. Levy, and J. E. Durbin.** 1998. The role of interferon in influenza virus tissue tropism. J Virol. **72:**8550-8.
49. **Gardner, E. M., E. D. Bernstein, M. Dorfman, E. Abrutyn, and D. M. Murasko.** 1997. The age-associated decline in immune function of healthy individuals is not related to changes in plasma concentrations of beta-carotene, retinol, alpha-tocopherol or zinc. Mech Ageing Dev. **94:**55-69.
50. **Gerhard, W.** 2001. The role of the antibody response in influenza virus infection. Curr Top Microbiol Immunol. **260:**171-90.
51. **Girodon, F., P. Galan, A. L. Monget, M. C. Boutron-Ruault, P. Brunet-Lecomte, P. Preziosi, J. Arnaud, J. C. Manuguerra, and S. Herchberg.** 1999. Impact of trace elements and vitamin supplementation on immunity and infections in institutionalized elderly patients: a randomized controlled trial. MIN. VIT. AOX. geriatric network. Arch Intern Med. **159:**748-54.
52. **Globerson, A., and R. B. Effros.** 2000. Ageing of lymphocytes and lymphocytes in the aged. Immunol Today. **21:**515-521.
53. **Gon, Y., S. Hashimoto, S. Hayashi, T. Koura, K. Matsumoto, and T. Horie.** 1996. Lower serum concentrations of cytokines in elderly patients with pneumonia and the impaired production of cytokines by peripheral blood monocytes in the elderly. Clin Exp Immunol. **106:**120-6.
54. **Goodwin, J. S., and P. J. Garry.** 1988. Lack of correlation between indices of nutritional status and immunologic function in elderly humans. J Gerontol. **43:**M46-9.
55. **Goodwin, J. S., and P. J. Garry.** 1983. Relationship between megadose vitamin supplementation and immunological function in a healthy elderly population. Clin Exp Immunol. **51:**647-53.
56. **Gotch, F., A. McMichael, and J. Rothbard.** 1988. Recognition of influenza A matrix protein by HLA-A2-restricted cytotoxic T lymphocytes. Use of analogues to orientate the matrix peptide in the HLA-A2 binding site. J Exp Med. **168:**2045-57.
57. **Gregory, V., M. Bennett, M. H. Orkhan, S. Al Hajjar, N. Varsano, E. Mendelson, M. Zambon, J. Ellis, A. Hay, and Y. P. Lin.** 2002. Emergence of influenza A H1N2 reassortant viruses in the human population during 2001. Virology. **300:**1-7.

58. **Grimble, R. F.** 1997. Effect of antioxidative vitamins on immune function with clinical applications. Int J Vitam Nutr Res. **67:**312-20.
59. **Grimble, R. F.** 1999. Nutritional influences on inflammation. Nestle Nutr Workshop Ser Clin Perform Programme. **2:**63-77.
60. **Grmek, M. D.** 1893. Les Maladies a l'Aube de la Civilization Accidentale. Payot, Paris.
61. **Gromme, M., and J. Neefjes.** 2002. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Mol Immunol. **39:**181-202.
62. **Guan, Y., K. F. Shortridge, S. Krauss, P. S. Chin, K. C. Dyrting, T. M. Ellis, R. G. Webster, and M. Peiris.** 2000. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. J Virol. **74:**9372-80.
63. **Guan, Y., K. F. Shortridge, S. Krauss, and R. G. Webster.** 1999. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci U S A. **96:**9363-7.
64. **Guo, H. C., T. S. Jardetzky, T. P. Garrett, W. S. Lane, J. L. Strominger, and D. C. Wiley.** 1992. Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. Nature. **360:**364-6.
65. **Hannet, I., F. Erkeller-Yuksel, P. Lydyard, V. Deneys, and M. DeBruyere.** 1992. Developmental and maturational changes in human blood lymphocyte subpopulations. Immunol Today. **13:**215, 218.
66. **Harling-McNabb, L., G. Deliyannis, D. C. Jackson, S. Gerondakis, G. Grigoriadis, and L. E. Brown.** 1999. Mice lacking the transcription factor subunit Rel can clear an influenza infection and have functional anti-viral cytotoxic T cells but do not develop an optimal antibody response. Int Immunol. **11:**1431-9.
67. **Hers, J. F.** 1966. Disturbances of the ciliated epithelium due to influenza virus. Am Rev Respir Dis. **93:**Suppl:162-77.
68. **Hill, D. A., S. Baron, J. C. Perkins, M. Worthington, J. E. Van Kirk, J. Mills, A. Z. Kapikian, and R. M. Chanock.** 1972. Evaluation of an interferon inducer in viral respiratory disease. Jama. **219:**1179-84.
69. **Hirsch, A.** 1883. Handbook of Geographical and Historical Pathology. New Sydenham Society, London.
70. **Hogan, R. J., E. J. Usherwood, W. Zhong, A. A. Roberts, R. W. Dutton, A. G. Harmsen, and D. L. Woodland.** 2001. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. J Immunol. **166:**1813-22.
71. **Horimoto, T., and Y. Kawaoka.** 2001. Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev. **14:**129-49.
72. **Huet, S., D. F. Nixon, J. B. Rothbard, A. Townsend, S. A. Ellis, and A. J. McMichael.** 1990. Structural homologies between two HLA B27-restricted

peptides suggest residues important for interaction with HLA B27. Int Immunol. **2:**311-6.
73. **Ivanova, R., N. Henon, V. Lepage, D. Charron, E. Vicaut, and F. Schachter.** 1998. HLA-DR alleles display sex-dependent effects on survival and discriminate between individual and familial longevity. Hum Mol Genet. **7:**187-94.
74. **Jameson, J., J. Cruz, and F. A. Ennis.** 1998. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. J Virol. **72:**8682-9.
75. **Jameson, J., J. Cruz, M. Terajima, and F. A. Ennis.** 1999. Human CD8+ and CD4+ T lymphocyte memory to influenza A viruses of swine and avian species. J Immunol. **162:**7578-83.
76. **Julkunen, I., K. Melen, M. Nyqvist, J. Pirhonen, T. Sareneva, and S. Matikainen.** 2000. Inflammatory responses in influenza A virus infection. Vaccine. **19:**S32-7.
77. **Justewicz, D. M., P. C. Doherty, and R. G. Webster.** 1995. The B-cell response in lymphoid tissue of mice immunized with various antigenic forms of the influenza virus hemagglutinin. J Virol. **69:**5414-21.
78. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol. **63:**4603-8.
79. **Keen, C. L., and M. E. Gershwin.** 1990. Zinc deficiency and immune function. Annu Rev Nutr. **10:**415-31.
80. **Kopf, M., B. Abel, A. Gallimore, M. Carroll, and M. F. Bachmann.** 2002. Complement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection. Nat Med. **8:**373-8.
81. **Kurtz, J., R. J. Manvell, and J. Banks.** 1996. Avian influenza virus isolated from a woman with conjunctivitis. Lancet. **348:**901-2.
82. **Lesourd, B., and L. Mazari.** 1999. Nutrition and immunity in the elderly. Proc Nutr Soc. **58:**685-95.
83. **Lesourd, B. M., M. Favre-Berrone, and M. Thiollet.** 1990. Action immunostimulante d'une supplementation orale complete chez des sujets ages denutris. Age and Nutrition. **1:**132-138.
84. **Lesourd, B. M., L. Mazari, and M. Ferry.** 1998. The role of nutrition in immunity in the aged. Nutr Rev. **56:**S113-125.
85. **Ligthart, G. J., J. X. Corberand, C. Fournier, P. Galanaud, W. Hijmans, B. Kennes, H. K. Muller-Hermelink, and G. G. Steinmann.** 1984. Admission criteria for immunogerontological studies in man: the SENIEUR protocol. Mech Ageing Dev. **28:**47-55.
86. **Lindsay, M. I., Jr., E. C. Herrmann, Jr., G. W. Morrow, Jr., and A. L. Brown, Jr.** 1970. Hong Kong influenza: clinical, microbiologic, and pathologic features in 127 cases. Jama. **214:**1825-32.
87. **Louria, D. B., H. L. Blumenfeld, J. T. Ellis, E. D. Kilbourne, and D. E. Rogers.** 1959. Studies on influenza in the pandemic of 1957-58. II. Pulmonary complications of influenza. J Clin Invest. **38:**213-265.

88. **Man, S., M. H. Newberg, V. L. Crotzer, C. J. Luckey, N. S. Williams, Y. Chen, E. L. Huczko, J. P. Ridge, and V. H. Engelhard.** 1995. Definition of a human T cell epitope from influenza A non-structural protein 1 using HLA-A2.1 transgenic mice. Int Immunol. **7:**597-605.
89. **Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador.** 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature. **409:**1055-60.
90. **Matikainen, S., J. Pirhonen, M. Miettinen, A. Lehtonen, C. Govenius-Vintola, T. Sareneva, and I. Julkunen.** 2000. Influenza A and sendai viruses induce differential chemokine gene expression and transcription factor activation in human macrophages. Virology. **276:**138-47.
91. **Matsukura, S., F. Kokubu, H. Noda, H. Tokunaga, and M. Adachi.** 1996. Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. J Allergy Clin Immunol. **98:**1080-7.
92. **Mbawuike, I. N., C. L. Acuna, K. C. Walz, R. L. Atmar, S. B. Greenberg, and R. B. Couch.** 1997. Cytokines and impaired CD8+ CTL activity among elderly persons and the enhancing effect of IL-12. Mech Ageing Dev. **94:**25-39.
93. **Mbawuike, I. N., A. R. Lange, and R. B. Couch.** 1993. Diminished influenza A virus-specific MHC class I-restricted cytotoxic T lymphocyte activity among elderly persons. Viral Immunol. **6:**55-64.
94. **McKenzie, R. C., T. S. Rafferty, and G. J. Beckett.** 1998. Selenium: an essential element for immune function. Immunol Today. **19:**342-5.
95. **McLachlan, J. A., C. D. Serkin, K. M. Morrey-Clark, and O. Bakouche.** 1995. Immunological functions of aged human monocytes. Pathobiology. **63:**148-59.
96. **McMichael, A.** 1994. Cytotoxic T lymphocytes specific for influenza virus. Curr Top Microbiol Immunol. **189:**75-91.
97. **McMichael, A. J., and B. A. Askonas.** 1978. Influenza virus-specific cytotoxic T cells in man; induction and properties of the cytotoxic cell. Eur J Immunol. **8:**705-11.
98. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare.** 1983. Cytotoxic T-cell immunity to influenza. N Engl J Med. **309:**13-17.
99. **McMichael, A. J., C. A. Michie, F. M. Gotch, G. L. Smith, and B. Moss.** 1986. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. J Gen Virol. **67:**719-26.
100. **Meydani, S. N., M. Meydani, J. B. Blumberg, L. S. Leka, G. Siber, R. Loszewski, C. Thompson, M. C. Pedrosa, R. D. Diamond, and B. D. Stollar.** 1997. Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. Jama. **277:**1380-6.
101. **Meydani, S. N., J. D. Ribaya-Mercado, R. M. Russell, N. Sahyoun, F. D. Morrow, and S. N. Gershoff.** 1991. Vitamin B-6 deficiency impairs interleukin 2

production and lymphocyte proliferation in elderly adults. Am J Clin Nutr. **53:**1275-80.

102. **Miller, R. A.** 1998. Aging and immune function, p. 947-966. *In* W. E. Paul, and W. E. Lippincott (eds), Fundamental Immunology. Williams & Williams, Philadelphia.

103. **Miller, R. A.** 1996. The aging immune system: primer and prospectus. Science. **273:**70-74.

104. **Mo, A. X., S. F. van Lelyveld, A. Craiu, and K. L. Rock.** 2000. Sequences that flank subdominant and cryptic epitopes influence the proteolytic generation of MHC class I-presented peptides. J Immunol. **164:**4003-10.

105. **Moser, M. R., T. R. Bender, H. S. Margolis, G. R. Noble, A. P. Kendal, and D. G. Ritter.** 1979. An outbreak of influenza aboard a commercial airliner. Am J Epidemiol. **110:**1-6.

106. **Mozdzanowska, K., M. Furchner, K. Maiese, and W. Gerhard.** 1997. CD4+ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. Virology. **239:**217-25.

107. **Mullbacher, A., M. Lobigs, J. W. Yewdell, J. R. Bennink, R. Tha Hla, and R. V. Blanden.** 1999. High peptide affinity for MHC class I does not correlate with immunodominance. Scand J Immunol. **50:**420-6.

108. **Murphy, B. R., S. Baron, E. G. Chalhub, C. P. Uhlendorf, and R. M. Chanock.** 1973. Temperature-sensitive mutants of influenza virus. IV. Induction of interferon in the nasopharynx by wild-type and a temperature-sensitive recombinant virus. J Infect Dis. **128:**488-493.

109. **Murphy, B. R., and M. L. Clements.** 1989. The systemic and mucosal immune response of humans to influenza A virus. Curr Top Microbiol Immunol. **146:**107-16.

110. **Murphy, B. R., J. A. Kasel, and R. M. Chanock.** 1972. Association of serum anti-neuraminidase antibody with resistance to influenza in man. N Engl J Med. **286:**1329-32.

111. **Murphy, B. R., D. L. Nelson, P. F. Wright, E. L. Tierney, M. A. Phelan, and R. M. Chanock.** 1982. Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. Infect Immun. **36:**1102-8.

112. **Murphy, B. R., and R. G. Webster** 1996. Orthomyxoviruses, p. 1397-1445. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (eds), Field virology, 3rd ed. Lippencott-Raven, Philadelphia, Pa.

113. **Neefjes, J.** 1999. CIIV, MIIC and other compartments for MHC class II loading. Eur J Immunol. **29:**1421-5.

114. **Nicholson, K. G.** 1998. Human Influenza, p. 219-264. *In* K. G. Nicholson, R. G. Webster, and A. J. Hay (eds), Textbook of Influenza. Blackwell Science Ltd, Oxford.

115. **Pahlavani, M. A.** 1998. T cell signaling: effect of age. Front Biosci. **3:**D1120-33.

116. **Pawelec, G., R. B. Effros, C. Caruso, E. Remarque, Y. Barnett, and R. Solana.** 1999. T cells and aging (update february 1999). Front Biosci. **4:**D216-69.
117. **Penn, N. D., L. Purkins, J. Kelleher, R. V. Heatley, B. H. Mascie-Taylor, and P. W. Belfield.** 1991. The effect of dietary supplementation with vitamins A, C and E on cell- mediated immune function in elderly long-stay patients: a randomized controlled trial. Age Ageing. **20:**169-74.
118. **Potter, C. W., and J. S. Oxford.** 1979. Determinants of immunity to influenza infection in man. Br Med Bull. **35:**69-75.
119. **Powers, D. C.** 1993. Influenza A virus-specific cytotoxic T lymphocyte activity declines with advancing age. J Am Geriatr Soc. **41:**1-5.
120. **Powers, D. C., and R. B. Belshe.** 1993. Effect of age on cytotoxic T lymphocyte memory as well as serum and local antibody responses elicited by inactivated influenza virus vaccine. J Infect Dis. **167:**584-592.
121. **Provinciali, M., A. Montenovo, G. Di Stefano, M. Colombo, L. Daghetta, M. Cairati, C. Veroni, R. Cassino, F. Della Torre, and N. Fabris.** 1998. Effect of zinc or zinc plus arginine supplementation on antibody titre and lymphocyte subsets after influenza vaccination in elderly subjects: a randomized controlled trial. Age Ageing. **27:**715-22.
122. **Rea, I. M., and D. Middleton.** 1994. Is the phenotypic combination A1B8Cw7DR3 a marker for male longevity? J Am Geriatr Soc. **42:**978-83.
123. **Remarque, E., and G. Pawelec.** 1998. T cell immunosenescence and its clinical relevance in man. Rev Clin Geront. **8:**5-25.
124. **Remarque, E. J.** 1999. Influenza vaccination in elderly people. Exp Gerontol. **34:**445-52.
125. **Richman, D. D., B. R. Murphy, S. Baron, and C. Uhlendorf.** 1976. Three strains of influenza A virus (H3N2): interferon sensitivity in vitro and interferon production in volunteers. J Clin Microbiol. **3:**223-6.
126. **Rimmelzwaan, G. F., A. C. Boon, J. T. Voeten, E. G. Berkhoff, R. A. Fouchier, and A. D. Osterhaus.** 2003. Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. submitted for publication.
127. **Rimmelzwaan, G. F., J. C. de Jong, T. M. Bestebroer, A. M. van Loon, E. C. Claas, R. A. Fouchier, and A. D. Osterhaus.** 2001. Antigenic and genetic characterization of swine influenza A (H1N1) viruses isolated from pneumonia patients in The Netherlands. Virology. **282:**301-6.
128. **Rimmelzwaan, G. F., and A. D. Osterhaus.** 1995. Cytotoxic T lymphocyte memory: role in cross-protective immunity against influenza? Vaccine. **13:**703-5.
129. **Rock, K. L.** 1996. A new foreign policy: MHC class I molecules monitor the outside world. Immunol Today. **17:**131-7.
130. **Ronni, T., T. Sareneva, J. Pirhonen, and I. Julkunen.** 1995. Activation of IFN-alpha, IFN-gamma, MxA, and IFN regulatory factor 1 genes in influenza A virus-infected human peripheral blood mononuclear cells. J Immunol. **154:**2764-74.

131. **Rota, P. A., E. P. Rocha, M. W. Harmon, V. S. Hinshaw, M. G. Sheerar, Y. Kawaoka, N. J. Cox, and T. F. Smith.** 1989. Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza. J Clin Microbiol. **27:**1413-6.
132. **Scholtissek, C., H. Burger, O. Kistner, and K. F. Shortridge.** 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology. **147:**287-94.
133. **Semba, R. D.** 1999. Vitamin A and immunity to viral, bacterial and protozoan infections. Proc Nutr Soc. **58:**719-27.
134. **Serwold, T., F. Gonzalez, J. Kim, R. Jacob, and N. Shastri.** 2002. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. Nature. **419:**480-3.
135. **Smith, W., C. H. Andrewes, and P. P. Laidlaw.** 1933. A virus obtained from influenza patients. Lancet. **i:**66-68.
136. **Solana, R., M. C. Alonso, and J. Pena.** 1999. Natural killer cells in healthy aging. Exp Gerontol. **34:**435-43.
137. **Sprenger, H., R. G. Meyer, A. Kaufmann, D. Bussfeld, E. Rischkowsky, and D. Gemsa.** 1996. Selective induction of monocyte and not neutrophil-attracting chemokines after influenza A virus infection. J Exp Med. **184:**1191-6.
138. **Stulnig, T., C. Maczek, G. Bock, O. Majdic, and G. Wick.** 1995. Reference intervals for human peripheral blood lymphocyte subpopulations from 'healthy' young and aged subjects. Int Arch Allergy Immunol. **108:**205-10.
139. **Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox.** 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science. **279:**393-6.
140. **Sutton, J., S. Rowland-Jones, W. Rosenberg, D. Nixon, F. Gotch, X. M. Gao, N. Murray, A. Spoonas, P. Driscoll, M. Smith, and et al.** 1993. A sequence pattern for peptides presented to cytotoxic T lymphocytes by HLA B8 revealed by analysis of epitopes and eluted peptides. Eur J Immunol. **23:**447-53.
141. **Takata, H., M. Suzuki, T. Ishii, S. Sekiguchi, and H. Iri.** 1987. Influence of major histocompatibility complex region genes on human longevity among Okinawan-Japanese centenarians and nonagenarians. Lancet. **2:**824-6.
142. **Talbott, M. C., L. T. Miller, and N. I. Kerkvliet.** 1987. Pyridoxine supplementation: effect on lymphocyte responses in elderly persons. Am J Clin Nutr. **46:**659-64.
143. **Tamura, S., T. Iwasaki, A. H. Thompson, H. Asanuma, Z. Chen, Y. Suzuki, C. Aizawa, and T. Kurata.** 1998. Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. J Gen Virol. **79:**291-9.
144. **Taylor, P. M., and B. A. Askonas.** 1986. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. Immunology. **58:**417-20.

145. **Taylor, R. M.** 1951. A further note on 1233 ('influenza C') virus. Archiv gesamte Virusforschung. **4:**485-500.
146. **Topham, D. J., and P. C. Doherty.** 1998. Clearance of an influenza A virus by CD4+ T cells is inefficient in the absence of B cells. J Virol. **72:**882-5.
147. **Tourdot, S., and K. G. Gould.** 2002. Competition between MHC class I alleles for cell surface expression alters CTL responses to influenza A virus. J Immunol. **169:**5615-21.
148. **Townsend, A. R., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael.** 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell. **44:**959-68.
149. **Trojan, A., M. Urosevic, J. Hummerjohann, R. Giger, U. Schanz, and R. A. Stahel.** 2003. Immune Reactivity Against a Novel HLA-A3-Restricted Influenza Virus Peptide Identified by Predictive Algorithms and Interferon-gamma Quantitative PCR. J Immunother. **26:**41-6.
150. **Tussey, L. G., S. Rowland-Jones, T. S. Zheng, M. J. Androlewicz, P. Cresswell, J. A. Frelinger, and A. J. McMichael.** 1995. Different MHC class I alleles compete for presentation of overlapping viral epitopes. Immunity. **3:**65-77.
151. **Utsuyama, M., K. Hirokawa, C. Kurashima, M. Fukayama, T. Inamatsu, K. Suzuki, W. Hashimoto, and K. Sato.** 1992. Differential age-change in the numbers of CD4+CD45RA+ and CD4+CD29+ T cell subsets in human peripheral blood. Mech Ageing Dev. **63:**57-68.
152. **Villadangos, J. A., R. A. Bryant, J. Deussing, C. Driessen, A. M. Lennon-Dumenil, R. J. Riese, W. Roth, P. Saftig, G. P. Shi, H. A. Chapman, C. Peters, and H. L. Ploegh.** 1999. Proteases involved in MHC class II antigen presentation. Immunol Rev. **172:**109-20.
153. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J Virol. **74:**6800-6807.
154. **Walsh, J. J., L. F. Dietlein, F. N. Low, G. E. Burch, and W. J. Mogabgab.** 1961. Bronchotracheal response in human influenza. Archiv Intern Med. **108:**376-388.
155. **Wang, W., C. M. Higuchi, and R. Zhang.** 1997. Individual and combinatory effects of soy isoflavones on the in vitro potentiation of lymphocyte activation. Nutr Cancer. **29:**29-34.
156. **Webster, R. G., J. A. Kasel, R. B. Couch, and W. G. Laver.** 1976. Influenza virus subunit vaccines. II. Immunogenicity and original antigenic sin in humans. J Infect Dis. **134:**48-58.
157. **Webster, R. G., G. B. Sharp, and E. C. Claas.** 1995. Interspecies transmission of influenza viruses. Am J Respir Crit Care Med. **152:**S25-30.

158. **Webster, R. G., K. F. Shortridge, and Y. Kawaoka.** 1997. Influenza: interspecies transmission and emergence of new pandemics. FEMS Immunol Med Microbiol. **18:**275-9.
159. **Wedemeyer, H., E. Mizukoshi, A. R. Davis, J. R. Bennink, and B. Rehermann.** 2001. Cross-reactivity between hepatitis C virus and Influenza A virus determinant-specific cytotoxic T cells. J Virol. **75:**11392-400.
160. **Wentworth, D. E., B. L. Thompson, X. Xu, H. L. Regnery, A. J. Cooley, M. W. McGregor, N. J. Cox, and V. S. Hinshaw.** 1994. An influenza A (H1N1) virus, closely related to swine influenza virus, responsible for a fatal case of human influenza. J Virol. **68:**2051-8.
161. **Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed.** 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. **77:**4911-27.
162. **Woodland, D. L., R. J. Hogan, and W. Zhong.** 2001. Cellular immunity and memory to respiratory virus infections. Immunol Res. **24:**53-67.
163. **Xu, X., C. B. Smith, B. A. Mungall, S. E. Lindstrom, H. E. Hall, K. Subbarao, N. J. Cox, and A. Klimov.** 2002. Intercontinental circulation of human influenza A(H1N2) reassortant viruses during the 2001-2002 influenza season. J Infect Dis. **186:**1490-3.
164. **Yewdell, J., L. C. Anton, I. Bacik, U. Schubert, H. L. Snyder, and J. R. Bennink.** 1999. Generating MHC class I ligands from viral gene products. Immunol Rev. **172:**97-108.
165. **Yewdell, J. W., and J. R. Bennink.** 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu Rev Immunol. **17:**51-88.
166. **Yewdell, J. W., J. R. Bennink, G. L. Smith, and B. Moss.** 1985. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc Natl Acad Sci U S A. **82:**1785-9.
167. **Zhang, R., Y. Li, and W. Wang.** 1997. Enhancement of immune function in mice fed high doses of soy daidzein. Nutr Cancer. **29:**24-8.
168. **Zheng, B., S. Han, Y. Takahashi, and G. Kelsoe.** 1997. Immunosenescence and germinal center reaction. Immunol Rev. **160:**63-77.
169. **Zissel, G., M. Schlaak, and J. Muller-Quernheim.** 1999. Age-related decrease in accessory cell function of human alveolar macrophages. J Investig Med. **47:**51-6.

Chapter 2

The magnitude and specificity of influenza A virus-specific CTL responses in humans is related to HLA-A and -B phenotype

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Journal of Virology 2002;76(2):582-590

Abstract

The repertoire of human cytotoxic T lymphocytes (CTL) in response to influenza A viruses has been shown to be directed towards multiple epitopes, with a dominant response to the HLA-A2-restricted $M1_{58-66}$ epitope. These studies, however, were performed with peripheral blood mononuclear cells (PBMC) of individuals selected randomly with respect to HLA phenotype or selected for the expression of one HLA allele without considering an influence of other HLA molecules. In addition, little information is available on the influence of HLA makeup on the overall CTL response against influenza viruses. Here, the influenza A virus-specific CTL response was investigated in groups of HLA-A and -B identical individuals. Between groups, the individuals shared three of the four HLA-A and -B alleles. After *in vitro* stimulation of PBMC with influenza virus, the highest CTL activity was found in HLA-A2 positive donors. A similar pattern was observed for the precursor frequency of virus-specific CTL (CTLp) *ex vivo*, with a higher CTLp frequency in HLA-A2+ donors than in HLA-A2 negative donors, which were unable to recognize the immunodominant $M1_{58-66}$ epitope. In addition, CTL activity and frequency of CTLp for the individual influenza virus epitopes were determined. The frequency of CTLp specific for the HLA-B8-restricted epitope $NP_{380-388}$ was three-fold lower in HLA-B27+ donors than in HLA-B27 negative donors. In addition, the frequency of CTLp specific for the HLA-A1-restricted epitope NP_{44-52} was three-fold higher in HLA-A1-, A2-, B8-, -B35-positive donors than in other donors, which was confirmed by measuring the CTL activity *in vitro*. These findings indicate that the epitope specificity of the CTL response is related to the phenotype of the other HLA molecules. Furthermore, the magnitude of the influenza virus-specific CTL response seems dependent on the HLA-A and -B phenotype.

Introduction

Influenza viruses are negative-sense RNA viruses that cause annual epidemics in the human population. Vaccination against influenza virus, aiming at the induction of virus neutralizing antibodies specific for the hemagglutinin (HA) and neuraminidase (NA) proteins, induces protective immunity when the HA and NA proteins of the vaccine strains closely resemble those of the circulating virus strains. Mutation of these proteins can result in viral escape from neutralizing antibodies (antigenic drift). In addition, occasionally new potentially pandemic influenza viruses emerge with novel HA and NA proteins, against which preexisting antibodies are absent in the human population (antigenic shift). In these cases, cytotoxic T lymphocytes (CTL) directed to more conserved internal proteins, such as nucleoprotein (NP), matrix protein and polymerase proteins may contribute to protective immunity against these pandemic viruses (25, 32, 37, 49), although changes in these internal proteins causing loss of CTL recognition have also been described (33, 51).
Influenza virus-specific CTL-mediated immunity has been investigated in both humans and mice. In mice, the CTL response was found to be directed to a limited number of epitopes. This ability of the immune system to focus the T cell responses to a limited number of epitopes is termed immunodominance (7, 8, 25, 52). In B6 mice the influenza A virus-specific CTL responses were directed against a number of H-2^b-restricted immunodominant epitopes (3, 5, 6). Likewise, after an infection of lymphocytic choriomeningitis virus (LCMV), the CTL response in mice was mainly directed against an epitope derived from the NP (50). Similar examples of immunodominance have been described for humans. In HLA-A2+ donors, the CTL response against influenza virus is predominantly directed to the HLA-A2-restricted epitope of the matrix protein (GILGFVFTL; $M1_{58-66}$) (2, 24, 25, 39). For Epstein-Barr virus (EBV) infections, it was reported that the cellular response is focused towards HLA-A11- and HLA-B8-restricted epitopes (23, 46). Nevertheless, other studies have indicated that the CTL response after both acute (influenza virus) and chronic (human immunodeficiency virus [HIV]) viral infections can be directed to a large number of epitopes (9, 28, 45).
Several factors have been reported to contribute to the phenomenon of immunodominance such as gamma interferon (IFN-γ) production, major histocompatibility complex (MHC) binding affinity of the epitopes, available T cell receptor repertoire, epitope abundance and antigen processing (1, 12, 13, 16, 21, 22, 38). However, the correlation between immunodominance and epitope-MHC binding affinity is presently under debate (15, 40). Several other factors that could possibly have an effect on immunodominance have not been investigated thoroughly, such as the influence of HLA phenotype, patient age, gender and history of infection. The first evidence regarding the involvement of MHC phenotype in immunodominance was observed by Doherty et al, describing in mice a minimization of the $H2D^b$ response in the presence of $H2K^k$ (18). Using modern tools for the measurement of CTL activity, these early findings were confirmed recently (4). In humans the role of HLA phenotype in immunodominance is still unclear. Tussey et al described the absence of a HLA-B8-restricted response against the influenza A virus $NP_{380-388}$ epitope in a HLA-B27+, HLA-B8+ donor (48), due to

competition for overlapping epitopes in the endoplasmatic reticulum by HLA-B8 and HLA-B*2702, resulting in the suboptimal loading of HLA-B8.
Although influenza virus-specific immunity mediated by CTL has been studied to a certain extent, in these studies peripheral blood mononuclear cells (PBMC) were obtained from donors selected for the presence of one particular HLA molecule, not controlling for HLA background, patient age or history of infection. In the present study CTL immunity towards influenza A virus and its individual epitopes was studied in donors with identical HLA-A and -B phenotypes and was compared with that of donors who share two or three out of the four HLA-A or -B alleles, enabling us to investigate the effect of HLA-phenotype on the specificity and the magnitude of the CTL response. To this end, the precursor frequency of CTL (CTLp) and CTL activity directed against influenza A virus and individual epitopes were determined *ex vivo* and after *in vitro* stimulation of PBMC with influenza virus, respectively.

Table 1: HLA-A and -B genotype of the 18 donors

	Donor	HLA-A*	HLA-B*
Group I	1	0101, 0201	0801, 3501
	2	0101. 0201	0801, 3501
	3	0101, 0201	0801, 3501
	4	0101, 0201	0801, 3501
Group II	5	0101, 0201	0801, 2705
	6	0101, 0201	0801, 2705
	7	0101, 0201	0801, 2705
	8	0101, 0201	0801, 2702
	9	0101, 0201	0801, 2705
Group III	10	0101, 0301	0801, 3501
	11	0101, 0301	0801, 3501
	12	0101, 0301	0801, 3501
	13	0101, 0301	0801, 3501
	14	0101, 0301	0801, 3501
	15	0101, 0301	0801, 3503
	16	0101, 0301	0801, 3503
Group IV	17	0201, 0302	2705, 3501
Group V	18	0201, 0301	0801, 3501

Material and Methods

Human subjects

A total of 18 healthy blood donors, between 35 and 50 years of age, were selected according to serological homology within the A-locus and B-locus of HLA class I

molecules (Tab. 1). Genetic subtyping of the HLA-A and HLA-B locus was performed using a commercial typing system (GenoVision, Vienna, Austria). PBMC were isolated by Lymphoprep® (Nycomed, Norway) gradient centrifugation and cryopreserved at -135°C. Serum samples were stored at -20°C and were used for serology (see below). All donors had serum antibodies against one or more influenza A virus strains (A/H1N1 or A/H3N2) measured by hemagglutination inhibition assay (HIA), indicative of one or more exposures to influenza virus in the past.

Serology
Serum samples were tested for the presence of influenza A virus-specific antibodies in the HIA according to standard methods (36, 41) using turkey erythrocytes. The sera were tested for antibodies against 10 influenza virus (A/H3N2) vaccine strains used since the emergence of these viruses in 1968, and for antibodies against 9 A/H1N1 strains, including the first isolate of H1N1 (A/Puerto Rico/8/34) and the latest H1N1 vaccine strain (A/New Caledonia/20/99). Ferret sera raised against the test antigens were used as positive controls.

Table 2: Influenza virus CTL epitopes used in this study

Epitope	Antigen (amino acid positions)	Restriction element	Reference
CTELKLSDY	NP (44-52)	HLA-A1	(17)
VSDGGPNLY	PB1 (591-599)	HLA-A1	(17)
GILGFVFTL	M1 (58-66)	HLA-A*0201	(2), (39)
AIMDKNIIL	NS1 (122-130)	HLA-A*0201	(33)
ILRGSVAHK	NP (265-273)	HLA-A3	(17)
ELRSRYWAI	NP (380-388)	HLA-B*0801	(47)
ADRGLLRDI [b]	NP (263-271)	HLA-B8	(44)
SRYWAIRTR	NP (383-391)	HLA-B*2705	(48), (27)
RRSGAAGAAVK	NP (174-184)	HLA-B27	(29)
ASCMGLIY	M1 128-125	HLA-B*3501	(19)

[b] Influenza B virus epitope

Preparation of B lymphoblastoid cell lines (BLCL)
Autologous EBV-transformed BLCL of each donor was established by culturing $1x10^6$ to $5x10^6$ PBMC in 1 ml culture supernatant from the EBV-producing cell line

S594 in 24-wells plates as previously described (43). No BLCL were established for donors 11 and 13.

Influenza virus and peptides

Sucrose gradient purified influenza A virus (H3N2) Resvir-9, a reassortant between A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2), was used for the infection of PBMC or BLCL. The virus was selected for this study since it contains all known influenza A virus CTL epitopes, in contrast to other virus strains (51). The infectious virus titer (10^9 50% tissue culture infectious dose) was determined in cell culture using Madin-Darby-Canine-Kidney (MDCK) cells as indicator cells, as described previously (42). All peptides (Tab. 2), corresponding to influenza A virus CTL epitopes, were manufactured, HPLC purified and analyzed by mass spectrometry (Eurogentec, Seraing, Belgium). Peptides were dissolved in DMSO at 5.0 μg/ml, diluted in RPMI 1640 (Life Technologies, Rockville, MD, USA) to 100 μM and stored at -20°C.

Infected PBMC for the stimulation of influenza virus-specific CTL

In order to validate the antigen-presenting capacity of PBMC, cells (10^6/ml) from two HLA-A2 negative, HLA-A1 positive donors were infected with influenza virus (Resvir-9) at a multiplicity of infection (MOI) of 3 in RPMI 1640 supplemented with 10% fetal calf serum, glutamin (2 mM), streptomycin (100 μg/ml), and penicillin (100 IU/ml) (R10F). After 1 h of incubation at 37°C, the cells were pelleted and resuspended in RPMI 1640 medium supplemented with 10% pooled human AB serum, glutamin (2mM), streptomycin (100 μg/ml), penicillin (100 IU/ml) and 2-mercaptoethanol (20 μM) (R10H) at a concentration of 2 x 10^6 cells/ml and distributed in a 96-wells U-bottom plate (100 μl). In duplicate wells, various numbers of cells of a HLA-A2+, HLA-A1-restricted T cell clone (A1/NP), specific for the influenza A virus NP_{44-52} epitope, were added and incubated for 5 h at 37°C. Next, GolgiStop (Pharmingen, San Diego, CA, USA) was added to each well and the cells were incubated for a subsequent 6 h at 37°C. The staining of intracellular IFN-γ was performed as recommended by the manufacturer, by using a Cytofix/Cytoperm kit (Pharmingen), phycoerythrin-conjugated anti-IFN-γ monoclonal antibody (MAb) (559326, Pharmingen), mouse anti-human-HLA-A2 MAb (Biotest AG, Dreieich, Germany) and a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse MAb (F0313, Dako, Glostrup, Denmark) at previously determined optimal MAb concentrations. The percentage HLA-A2+, IFN-γ+ cells of the A1/NP clone was determined by flow cytometry. A1/NP stimulated with peptide loaded BLCL and uninfected PBMC were included as positive and negative controls respectively. The assay was done in duplicate and the results were calculated from the averages of duplicate wells.

In vitro stimulation of PBMC with influenza virus

PBMC were resuspended at 10^6/ml in R10F and infected with influenza A virus (Resvir-9) at an MOI of 3 for 1 h at 37°C. After centrifugation the stimulator PBMC were resuspended in R10H and added to uninfected responder PBMC in a 25-cm^2-diameter flask at a ratio of 1:1, which was found to be optimal. A total of 10 x 10^6

PBMC were incubated for 2 days at 37°C before recombinant interleukin-2 was added (50 U/ml). Following a subsequent 7-day incubation at 37°C the cells were harvested, analyzed by flow cytometry, and used as effector cells in the CTL assay.

CTL assay

HLA-A- and –B-matched BLCL (10^6) were incubated in R10F in the presence of 5 μM peptide (Tab. 2) and were used as target cells. In addition, BLCL were infected with influenza A virus (Resvir-9) at an MOI of 1 or were left untreated and used as positive and negative controls, respectively. In order to rule out any bystander activation, every CTL assay contained target cells loaded with an HLA-B8-restricted influenza B virus epitope ($NP_{263\text{-}271}$, Tab. 2). After incubation for 16 hr at 37°C, target cells were washed once in serum-free medium and 5 x 10^5 target cells were labeled for 1 h at 37°C with 50 μCi of $Na_2[^{51}Cr]O_4$ in RPMI 1640 medium. The cells were washed three times in R10F and resuspended at 10^5 cells/ml. Effector cells were transferred to 96-well V-bottom plates at a concentration of 1 x 10^5 and 5 x 10^4 cells/100 μl and 50 μl of the different target cells were added (effector:target cell ratio ([E:T-ratio] of 20:1 and 10:1). Furthermore, target cells were lysed with 100 μl 10% Triton X-100 or incubated with R10F to determine the maximum and spontaneous release. Following 4 h of incubation at 37°C, 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)] x 100. The data are presented as the average specific lysis of at least three wells.

Detection of CTL specific for individual influenza virus epitopes by ELIspot assay

A 96-well Silent Screen Plate (Life Technologies) was coated with 7.5 μg/ml of anti-IFN-γ MAb 1-DIK (Mabtech, Stockholm, Sweden) in 100 μl sodium-bicarbonate buffer (0.1 M) overnight at 4°C. The plates were washed three times with PBS and blocked with R10H for 2 h at 37°C. For the detection of epitope-specific CD8+ T cells, PBMC were incubated at a density of 2.5 x 10^5 cells/well in 150 μl R10H in the presence of HLA-compatible peptides (10 μM) in quadruplicate wells. The specificity of the ELIspot assay for the enumeration of epitope-specific CD8+ T cells was previously validated by others (31). Cells treated with phytohemagglutinin (1 μg/ml) (Roche Diagnostics, Mannheim, Germany) and untreated cells were used as positive and negative controls respectively. After 6 h at 37°C the wells were washed six times with PBS-0.05% Tween 20 (PBST) (Sigma Chemical Co., St. Louis, MO, USA). Detection of secreted IFN-γ was done with 100 μl of biotinylated anti-IFN-γ MAb 7-B6-1 (1.0 μg/ml, Mabtech) overnight at 4°C. After washing the plates three times with PBST, 100 μl of a 1:1000 diluted streptavidin labeled with alkalin phosphatase was added for 1 h at room temperature. The plates were subsequently washed three times and 100 μl of a phosphatase substrate BCIP/NBT (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was added per well. After an incubation of 1 h at room temperature the reaction was terminated by washing the plates three times with water. The frequency of peptide-

specific CTLp was based on the number of spots obtained in two independent experiments, counted by two individuals, and given as the number of CTLp per 2.5 x 10^5 PBMC for peptide-specific cells or percentage of CTLp within the CD8+ T cell fraction.

Detection of virus-specific CTL by ELIspot assay
Since it has been shown that NK cells are specifically activated by influenza virus-infected cells (34) it was necessary to isolate the CD8+ T lymphocytes, followed by the depletion of CD16+ cells, using Dynabeads (see below), in order to exclude IFN-γ spots produced by CD4+ T cells or NK cells. The CD3+ CD8+ CD16 negative cells (> 98% pure) were incubated with 4 x 10^4 autologous influenza A virus-infected BLCL or uninfected BLCL, for 90 min at 37°C in a 96-wells V-bottom plate following centrifugation for 1 min at 140 x g. For donor 11 and 13 no autologous BLCL were available and therefore HLA-A- and HLA-B-matched BLCL from donor 10 were used. A total number of 2 x 10^4 or 1 x 10^4 effector cells were used and the assay was performed in quadruplicate. After preincubation the cells were transferred to a plate coated with anti-IFN-γ MAb 1-DIK (see above) and incubated for an additional 5 h at 37°C. Virus-specific CD8+ T cells were visualized as described above, and the spots were counted by two individuals. The frequency of virus-specific CTLp was calculated from the number of

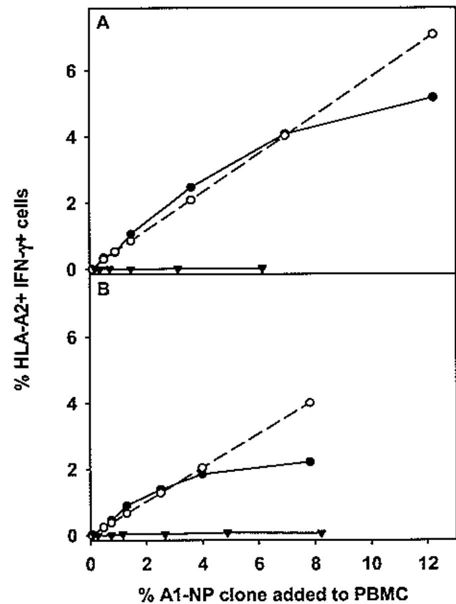

Figure 1: Ability of influenza virus-infected PBMC to activate an influenza virus-specific CTL clone.
Influenza virus-infected (Resvir-9, MOI = 3) or uninfected PBMC, from two HLA-A1-positive, HLA-A2-negative donors (A and B), were incubated in the presence of serially diluted HLA-A2+ A1/NP clone. The percentage of IFN-γ positive cells within the A1/NP population was determined by flow cytometry, using HLA-A2-specific antibodies (●). The calculated percentage IFN-γ-producing cells of A1/NP clone, determined after multiplying the maximal percentage IFN-γ positive A1/NP cells after stimulation with NP_{44-52} (5 μM)-loaded HLA-A1-positive, HLA-A2-negative BLCL (58.1%) with the percentage of cells of the A1/NP clone present within the total PBMC population, was plotted (dotted line). When the experimental value (percentage IFN-γ-positive cells) is similar to the calculated value, the virus-infected PBMC are able to stimulate the cells of the A1/NP clone. Uninfected PBMC did not induce IFN-γ production at any percentage of cells of the A1/NP clone (▼). The results represent the averages of duplicate wells

specific spots of multiple experiments and given as the percentage of influenza A virus-specific CD8+ T cells within the total CD8 population.

Characterization, depletion and isolation of T cells from PBMC

Depletion and isolation of CD4+ or CD8+ T cells was performed using DYNABEADS M-450 (111.05 and 111.07, Dynal, Oslo, Norway) according to manufacturer's protocol. The depletion of CD16+ cells was done with pan-anti-mouse immunoglobulin G-labeled Dynabeads (110.22, Dynal) coupled to mouse anti-human-CD16 MAb (30621A, Pharmingen). The success of the procedure was monitored by flow cytometry, using mouse MAb against human CD3 (CD3-RPE-Cy5; C7067; Dako), CD4 (CD4-RPE: F0805; Dako), CD8 (CD8-FITC: F0765; Dako) and CD16 (CD16-RPE: R7012; Dako). Flow cytometry was also used to determine the percentage CD3+, CD4+, and CD8+ cells in each donor before and after stimulation of PBMC with influenza virus.

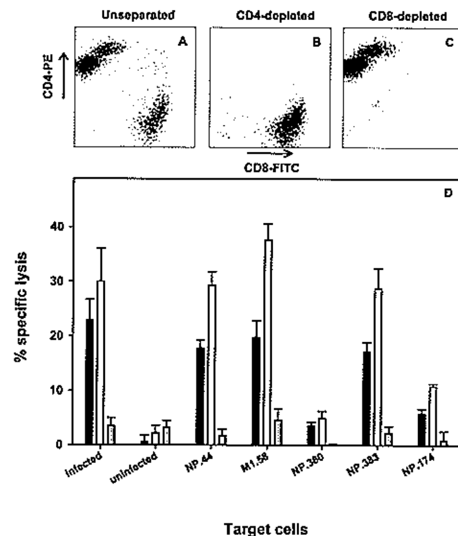

Figure 2: CD8+ T cells mediate CTL activity of PBMC after *in vitro* stimulation with influenza virus. Uninfected PBMC (donor 7) were stimulated *in vitro* with influenza virus-infected PBMC (Resvir-9, MOI = 3) at a ratio of 1:1, and virus-specific T cells were expanded for 9 days. Subsequently, the effector cells were divided into three portions; CD4 depleted (panel B and open bars in panel D), CD8 depleted (panel C and grey bars in panel D), or unseparated (panel A and black bars in panel D). Confirmation of depletion was done by flow cytometry (> 99% pure). The effector cells were added to $Na_2[^{51}Cr]O_4$-labelled target cells (BLCL), at an E:T-ratio of 10:1. BLCL were either infected with influenza A virus (Resvir-9, MOI = 1), left uninfected or pulsed with 5 μM HLA-matched viral peptide (as indicated). Results are given as the average of triplicate wells ± SD

Statistical analysis

Results are presented as mean ± standard deviations (SD). Statistical significance was determined using a Student *t* test and $P < 0.05$ was considered statistically significant. Donors with a genotypic mismatch for a certain HLA class I allele were excluded from the data analysis.

Results

Infected PBMC as antigen presenting cells for the stimulation of influenza virus-specific CTL

Using an HLA-A1-restricted T cell clone (A1/NP) specific for NP_{44-52}, the antigen-presenting capacity of influenza virus-infected PBMC was evaluated. First it was demonstrated that a maximum of 58.1% of the cells of the A1/NP clone were activated after stimulation with peptide-loaded HLA-A1+ BLCL. This percentage was used to calculate the expected percentage of IFN-γ-producing cells of the A1/NP clone after stimulation with virus-infected HLA-A1+ PBMC (figure 1, dotted line), which was compared with the percentage of cells that were actually activated (figure 1, solid line). Influenza virus-infected PBMC of donor A activated cells of the A1/NP clone to similar extend as peptide loaded BLCL when the A1/NP cells constituted up to 4% of the PBMC population (figure 1a). At higher cell numbers of the A1/NP clone the antigen-presenting capacity of infected PBMC was no longer sufficient for the activation of all A1/NP cells. Influenza virus-infected PBMC of donor B activated the A1/NP cells when they constituted up to 2% of the total PBMC population (figure 1b).

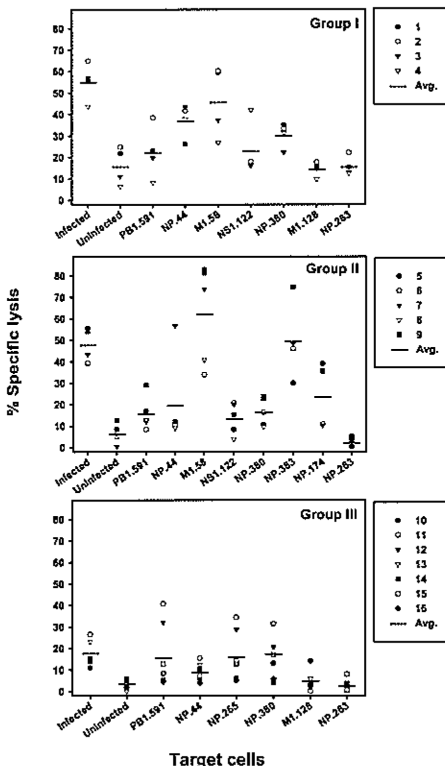

Figure 3: Influenza virus- and epitope-specific CTL activity in PBMC from donors with identical HLA-A and -B phenotypes. PBMC were stimulated with influenza virus, and the resulting effector cells were tested for CTL activity against radioactively labeled HLA-A- and -B-matched target cells (BLCL), either infected with influenza virus (Resvir-9, MOI = 1), left uninfected, or loaded with 5 μM peptide (as indicated) at an E:T-ratio of 20:1. The three groups correspond to HLA-A1-, -A2-, -B8-, -B35-positive donors (group I), HLA-A1-, -A2-, -B8-, -B27-positive donors (group II) and HLA-A1, -A3-, -B8-, -B35-positive donors (group III). The percentage of specific lysis was calculated from at least three wells and the average CTL activity for a given target cell within a group is plotted (dotted line). The CTL activity against virus-infected BLCL and HLA-B27 peptide-pulsed BLCL were not included for donor 8 (HLA-B*2702+). Furthermore, donors 15 and 16 (HLA-B*3503+) were excluded from the calculation of CTL activity specific for virus-infected BLCL and HLA-B*3501-restricted epitope $M1_{128-135}$-pulsed BLCL.

CD8+ phenotype of effector cells in CTL assays

To assess whether *in vitro*-expanded CD8+ T cells were responsible for the lysis of influenza A virus-infected or peptide-loaded target cells, PBMC of one donor were stimulated with influenza virus, followed by a CTL assay against HLA-matched targets cells infected with influenza virus or loaded with a number of different peptides. Prior to the CTL assay the effector cells were divided into three portions; one portion was used to deplete CD4+ T cells, from a second portion the CD8+ T cells were depleted, and a third portion was used as unseparated control cells. The depletion of either CD4+ or CD8+ T cells was confirmed by flow cytometry (figure 2). The three effector cell populations were added to target cells at an E:T ratio of 10:1. The lysis of virus-infected target cells and peptide-loaded target cells increased after depletion of CD4+ T cells compared to that of the unseparated control effector cell population. However, after depletion of CD8+ T cells it was found that the specific lysis of the target cells was reduced to background levels (figure 2), indicating that the CD8+ T cells were responsible for the lytic activity of virus-stimulated bulk cultures.

CTL responses to influenza virus and epitopes in HLA class I defined donors

The CTL responses specific for influenza A virus or viral epitopes were compared within a group of HLA-A and -B identical donors or between groups of donors mismatched for one HLA molecule. The percentage specific lysis for HLA-A1-, A2-, B8-, B35-positive donors revealed two donors (donor 1 and 2) with the highest percentage of specific lysis against target cells loaded with the HLA-A2-restricted $M1_{58-66}$ epitope (group I, figure 3). In donor 3 the highest response was directed towards NP_{44-52} (HLA-A1), while donor 4 demonstrated the highest response towards $NS1_{122-130}$, a second HLA-A2-restricted epitope. In the second group, containing HLA-A1-, -A2-, -B8-, -B27-positive donors, the response was dominated by the $M1_{58-66}$ epitope in four out of five donors (group II, figure 3). One donor (donor 6) showed a greater response against the HLA-B27-restricted $NP_{383-391}$ epitope. HLA-A1-, -A3-, -B8-, -B35-positive donors within group III (Tab. 1) demonstrated a more diverse response against the different epitopes tested. From the seven donors within group III, three donors showed the highest response to the HLA-B8-restricted epitope $NP_{380-388}$ (group III, figure 3). Furthermore, donor 14 had the highest response to the HLA-A3-restricted epitope $NP_{265-273}$, donor 10 had the highest response against the HLA-B35-restricted epitope $M1_{128-135}$ and finally donors 11 and 12 responded best to the HLA-A1-restricted epitope $PB1_{591-599}$. Of the remaining two donors, donor 17, an HLA-A2-, -A3-, -B8-, -B27-positive donor, had the highest percentage of specific lysis against the HLA-B27-restricted epitope $NP_{174-184}$, whereas donor 18, an HLA-A2-, -A3-, -B27-, -B35-positive donor, showed the highest response to the $NP_{383-391}$ epitope (data not shown). Overall, the $M1_{58-66}$-specific response was found to be dominant in seven out of 11 HLA-A2-positive donors compared with the response against the other epitopes. For HLA-A1, -A2 and -B27 the response to multiple peptides presented by one HLA molecule could be investigated. It was found that for HLA-A2 the response against $M1_{58-66}$ was higher than the response to $NS1_{122-130}$ in 10 out of 11 cases (figure 3). For the HLA-

B27-restricted epitopes (donor 8 excluded due to its HLA-B*2702 genotype), three donors demonstrated the highest response towards $NP_{383-391}$ and two donors to $NP_{174-184}$. Finally, the preferred recognition of the two HLA-A1-restricted epitopes, NP_{44-52} and $PB1_{591-599}$, was evenly distributed over the donors.
Comparison of the magnitude of the CTL response against virus-infected target cells between the groups of donors revealed a lower average percentage of specific lysis of target cells in group III ($P < 0.001$, figure 3). This inferior recognition of target cells was not caused by a difference in infectibility of the three target cells (data not shown). Therefore the lower percentage of specific lysis observed for donors within group III is likely to be caused by a reduction in the number of influenza virus-specific CTL after *in vitro* stimulation of PBMC or a reduced lytic capacity of the respective CTL. When the magnitude of the response against the different influenza

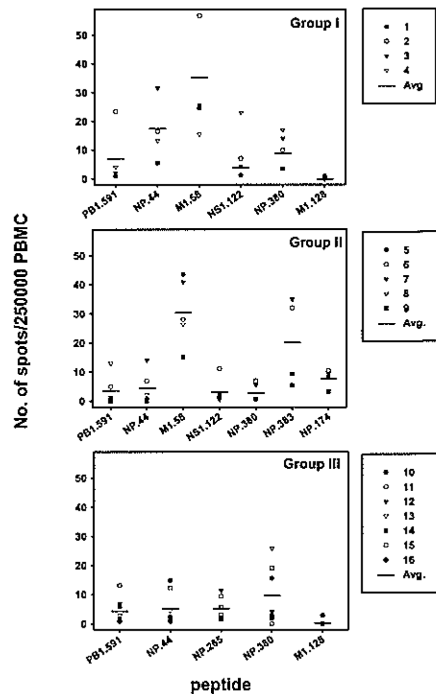

Figure 4: Frequency of CTLp specific for individual influenza virus epitopes.
PBMC from HLA-A and -B identical donors were tested for the frequency of influenza A virus epitope-specific CTLp *ex vivo* using an ELIspot assay. The graphs represent the different groups of donors; group I (HLA-A1, -A2, -B8, -B35), group II (HLA-A1, -A2, -B8, -B27) and group III (HLA-A1, -A3, -B8, -B35). The number of spots per 2.5×10^5 PBMC specific for each epitope is given for each donor individually and the average number of spots is given for the entire group (dotted line). The data are given as the average number of specific spots derived from two independent experiments of quadruplicate wells, counted by two individuals. Data from donor 8 (HLA-B*2702+) and donors 15 and 16 (HLA-B*3503+) were excluded for $NP_{383-391}$ and $M1_{128-135}$ respectively.

virus epitopes presented by shared HLA molecules (A1, B8) was compared between the three donor groups, no difference was found for the HLA-A1-restricted epitope $PB1_{591-599}$ (figure 3). The second HLA-A1-restricted epitope NP_{44-52} had a higher response in group I, than in both group II and III. The average percentage specific lysis specific for the HLA-B8-restricted $NP_{380-388}$ epitope was lower in the donors from group II (HLA-B27+) than in the donors from group I and III. The response against the only known HLA-B35 epitope was very low, and only two donors (10 and 13) showed a response (> 5% specific lysis) against the $M1_{128-135}$ epitope. As expected, none of the donors had a response against the HLA-B8-restricted

irrelevant influenza B virus epitope $NP_{263-271}$ after stimulation with influenza A virus (Resvir-9).

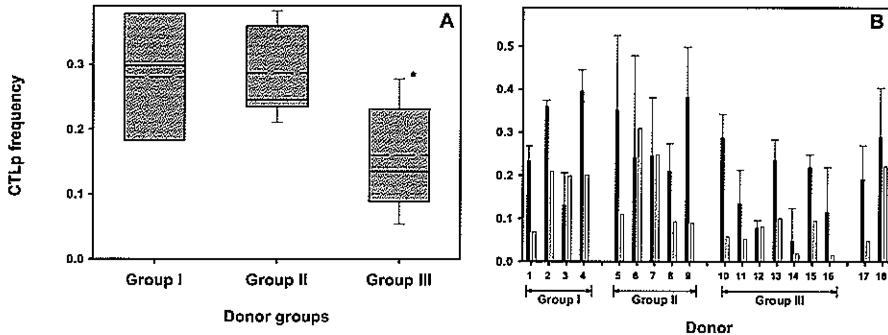

Figure 5: Lower influenza virus-specific CTLp frequencies in HLA-A2-negative donors. (A) The influenza A virus-specific CTLp frequency (presented as percentage virus-specific cells of CD8+ T cells), determined in ELIspot assay after stimulation with influenza virus-infected autologuous BLCL and uninfected autologuous BLCL, is plotted for each group of donors: group I (HLA-A1, -A2, -B8, -B35), group II (HLA-A1, -A2, -B8, -B27) and group III (HLA-A1, -A3, -B8, -B35). A significant difference (*, $P = 0.028$) was found between CTLp frequency specific for influenza virus in HLA-A2+, HLA-B27+ donors (group II) compared to that with HLA-A2-negative donors (group III). The box comprises 25th to 75th percentiles, while the error bars represent the 10th and 90th percentile from the average CTLp frequency (solid line). Median CTLp frequency is represented by a dotted line. (B) Representation of the contribution of CTLp frequency specific for individual peptides (open bars) to the total influenza virus-specific CTLp frequency (black bars). The percentage epitope-specific CTLp was calculated for each donor by dividing the sum of the specific spots, found for all epitopes by the number of CD8+ T cells within the PBMC (determined by flow cytometry). Results are given as the average percentages virus-specific CTLp frequency (black bars) calculated from 2 to 3 independently repeated experiments $\pm$ SD, whereas the epitope-specific CTLp frequency (open bars) was determined in two independently repeated experiments. Donors 11 and 13 were stimulated with HLA-matched BLCL from donor 10.

Frequencies of T cells specific for individual influenza virus epitopes in HLA-defined donors

The highest CTLp frequency in donors 1 and 2 was found to be specific for $M1_{58-66}$ (ranging from 24.6 to 57 per 2.5 x 10^5 PBMC, respectively) as determined by ELIspot assay. Donor 3 had the highest frequency of CTLp specific for NP_{44-52} (31 per 2.5 x 10^5 PBMC) and donor 4 had the highest frequency of CTLp specific for $NS1_{122-130}$ (23 per 2.5 x 10^5 PBMC) (figure 4). In donors of group II a dominance of the $M1_{58-66}$ epitope was found in four out of five donors, ranging from 15 spots per 2.5 x 10^5 PBMC in donor 9 to 43 spots per 2.5 x 10^5 PBMC in donor 5. Donor 6 showed a higher frequency of $NP_{383-391}$-specific CTL (32 per 2.5 x 10^5 PBMC). Five out of seven donors from group III showed a dominant response to the HLA-B8-

restricted epitope $NP_{380-388}$ (2 to 26 per 2.5 x 10^5 PBMC), while the remaining two donors showed the highest responses to the $PB1_{591-599}$ epitope (13 spots per 2.5 x 10^5 PBMC in donor 11) and the $NP_{265-273}$ epitope (12 spots per 2.5 x 10^5 PBMC in donor 12), respectively. In donor 17 the dominant response was directed to $NP_{174-184}$ (88 spots per 2.5 x 10^5 PBMC), while donor 18 had the highest number of spots after stimulation with $M1_{58-66}$ (13 spots per 2.5 x10^5 PBMC) (data not shown).
Comparison of the frequencies of CTLp specific for two epitopes presented by the same HLA class I molecule, showed that the HLA-A2-restricted epitope $M1_{58-66}$ dominated over $NS1_{122-130}$ in 10 out of 11 donors (figure 4). Within HLA-B27, a higher CTLp frequency was found specific for $NP_{383-391}$ than for $NP_{174-184}$ in three out of five donors (figure 4, data not shown). Finally, for the HLA-A1 molecule the NP_{44-52} epitope was dominantly recognized in 8 out of 16 donors and the $PB1_{591-599}$ epitope in 4 donors, and the remaining 4 donors had a similar response to both epitopes or no response at all (donor 9).
When the CTLp frequency against the different epitopes was compared between the three groups, a three-fold lower $NP_{380-388}$-specific CTLp frequency was found for the in HLA-B8+, HLA-B27+ donors (P = 0.09, group II), when compared with HLA-B8-positive, HLA-B27-negative donors (figure 4). Furthermore, the NP_{44-52}-specific CTLp frequency in donors from group I was three times higher then the CTLp frequency in donors from group II and III (P = 0.013). No difference in the CTLp frequency specific for $M1_{58-66}$, $PB1_{591-599}$ and $NS1_{122-130}$ was observed between the different groups. Finally, it was noted that the response to the HLA-B35-restricted epitope $M1_{128-135}$ was virtually absent in all donors.

Frequencies of influenza virus-specific T cells in HLA-defined donors

The sum of frequencies of CTLp specific for synthetic peptides representing known CTL epitopes was lower in HLA-A2-negative donors (group III) than in HLA-A2+ donors (groups I and II) ($P < 0.05$). However, since the whole repertoire of HLA class I-restricted epitopes for influenza A virus is not known, it is possible that in HLA-A2-negative donors a dominant response was elicited against unknown epitopes. To test whether this was the case or that indeed in HLA-A2-negative donors the CTL response towards influenza virus is less, the number of influenza A virus-specific CD8+ CTL was enumerated in all donors. In figure 5a the average percentage CTLp frequency is plotted for each of the three groups. In HLA-A1-, A2-, B8-, B35-positive donors (group I) 0.28% of the CD8+ T cells were influenza A virus-specific, and in HLA-A1-, -A2-, -B8-, -B27-positive donors (group II), 0.29% were found to be specific for influenza virus. Donors in group III (HLA-A1, -A3, -B8, -B35) had a significantly lower (P = 0.028) influenza virus-specific CTLp frequency (0.16%) compared to that of group II (figure 5a), but not compared to that of group I (P = 0.09). Comparison of all HLA-A2+ donors (including 17 and 18) with all HLA-A2-negative donors, found a significantly higher frequency of CTLp for influenza virus in HLA-A2+ donors (P = 0.011).
Comparison of the number of spots found after stimulation with whole virus with that found after stimulation with individual peptides revealed that the response against the individual peptides accounted for 12 to 100% of the total response against influenza virus (figure 5b). Four donors had a response against the epitopes

that was equal to the entire virus-specific response. Overall a correlation ($r = 0.8$; with the exception of two outliers, donor 3 and 10) was observed between frequencies of virus-specific CTLp and CTL activity (figure 6). Figure 6 also shows the difference in CTLp frequency and CTL activity between HLA-A2-positive and HLA-A2-negative donors.

Discussion

In this study it was shown that there is a relationship between HLA class I background and the specificity and magnitude of the CTL response specific for influenza A virus and its individual epitopes.

Prior to this study the *in vitro* stimulation protocol and the phenotype of the resulting effector cells were validated. It was concluded that influenza virus-infected PBMC were able to activate large numbers of cells of a CD8+ T cells clone (A1/NP) efficiently and quantitatively. In addition, the effector function was solely mediated by CD8+ T cells and the observed CTL activities were not the result of bystander activation, since the response towards an influenza B virus epitope ($NP_{263-271}$) was similar to the response against uninfected control cells.

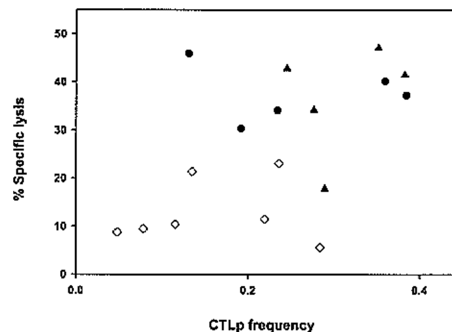

Figure 6: Correlation between CTL activity and influenza virus-specific CTLp frequency.
Virus-specific CTL activity was plotted, using influenza virus-infected HLA-matched BLCL at an E:T ratio of 20:1, against the frequency of virus-specific CTLp (presented as percentage virus-specific cells of CD8+ T cells), obtained from the ELIspot assays using influenza virus-infected autologous BLCL. All 18 donors were divided into several groups; HLA-A2-negative donors (◇), HLA-A2-positive, HLA-B27-negative (●) and HLA-A2-positive, HLA-B27-positive (▲). Two HLA-A2-negative donors (11 and 13) were stimulated with HLA-matched BLCL (donor 10). Donor 8 was excluded from the analysis.

The virus used for this study was a reassortant virus between A/Puerto Rico/8/34 and A/Nanchang/933/95, which contains the HA, NA and NP from the A/Nanchang/933/95 virus and all known CTL epitopes for influenza virus. The donors selected for this study were between 35 and 50 years of age, thereby controlling for age-related differences in immune reactivity and reducing the impact of the number of times the donors had been infected with influenza virus. All donors had been infected with influenza virus at least once, as indicated by the presence of virus-specific serum antibodies. Based on the age of the donors it can be expected, however, that they had been infected at least two or three times consecutively, which is important because it has been described that

responses against single epitopes differ between primary and secondary infections (10, 11).
The number of virus-specific CTLp *ex vivo* corresponded to approximately 0.1 to 0.5% of the total number of CD8+ T cells, which is within the normal range of percentage of IFN-γ+ influenza A virus-specific T cells measured by flow cytometry (unpublished observation). Comparison of the percentage virus-specific CTL between groups revealed a significant difference in CTLp frequency between HLA-A2-positive donors and HLA-A2-negative donors (figure 5 and 6), paralleled by a lower *in vitro* CTL activity against influenza virus-infected target cells in HLA-A2-negative donors. The clinical relevance of a lower frequency of virus-specific CTLp in individuals infected with influenza virus and whether a reduced CTLp frequency *in vivo* results in a similar reduction in specific CTL activity during virus infection remains unknown. This study is not the first to describe an association between magnitude of CTL response and HLA phenotype in humans. Disease severity in malaria, progression to AIDS in HIV infected individuals and human T cell lymphotropic type I associated myelopathy were reported to be associated with certain HLA molecules. However, these findings were mainly based on epidemiological data in heterogeneous cohorts of donors (20, 26, 30). In contrast, our study used a very well-defined cohort of HLA identical donors, enabling us to investigate the relationship between HLA phenotype and CTL response in detail.
HLA class I phenotype was found to influence the frequency of CTLp specific for individual influenza virus epitopes. The CTLp frequency specific for the HLA-A1-restricted epitope NP_{44-52} was significantly higher in HLA-A1-, -A2-, -B8-, -B35-positive donors (group I) than in both groups II and III (figure 4), resulting in a higher epitope-specific CTL activity following *in vitro* stimulation of PBMC with influenza virus. Since the higher CTLp frequency was observed in group I and not in groups II and III, it is difficult to link this difference to one HLA-A or -B molecule. Possibly, HLA-C or HLA class II molecules have influenced the outcome of the CTL response to this epitope. It was also noted that the frequency of CTLp specific for the HLA-B8-restricted epitope $NP_{380-388}$ was three-fold lower in HLA-B8+, HLA-B27+ donors (group II). This reduction in frequency of cells specific for the $NP_{380-388}$ peptide in HLA-B27+ donors can be explained by the presentation of overlapping peptides, as described previously by Tussey *et al* (48). However, after *in vitro* stimulation with influenza virus, the difference in CTL activity, specific for the $NP_{380-388}$ epitope, between HLA-B27-positive and HLA-B27-negative donors was less pronounced.
From the peptide-specific responses it was concluded that the CTL response against influenza virus is multispecific and directed against a number of different epitopes. On average 50% (ranging from 12% to 100%) of the virus-specific response is accounted for by known viral epitopes, indicating that in some donors more unknown epitopes are recognized in the response to influenza virus infections. The variability of the peptide-specific responses indicates that these data should be used with caution for the interpretation of the virus-specific CTL response against all epitopes (9). CTL activity in individual donors ranged from specificity towards all known peptides or two peptides, independent of HLA background. The observation

that in humans the influenza virus-specific CTL response is directed to multiple epitopes is in agreement with previously described studies (24, 28).
The dominance of the HLA-A2-restricted epitope $M1_{58-66}$ (24) is less strict than previously thought. Although all donors had a response to this epitope, 4 out of 11 HLA-A2+ donors showed a greater response against another epitope. In each of these four cases the dominant epitope was different. This finding adds to a study by Martinon, describing a greater response against an HLA-B37-restricted epitope than the HLA-A2 epitope $M1_{58-66}$ (35). It was also noted that in several cases the CTL response restricted to one HLA class I molecule was directed to both epitopes equally, indicating that there was no immunodominant response to one of the two epitopes. A low CTL response towards the HLA-B35-restricted epitope $M1_{128-135}$ was detected in 2 out of 13 HLA-B35+ donors. A possible explanation for the low reactivity against this peptide is the cysteine residue within the epitope, enabling the epitope to form dimers, and therefore reduce the ability to bind to the HLA molecule (14). However, freshly diluted peptide loaded onto target cells for 1 h did not result in a higher CTL activity. A second possibility is the relatively low binding affinity of the epitope to the HLA-molecule (19). Finally, the donors selected for this study can have an undetectable frequency of $M1_{128-135}$-specific CTL.
To our knowledge this is the first systematic study in which influenza virus-specific CTL responses are compared in groups of donors of well-defined HLA phenotype. The data presented here indicate that the HLA class I background of individuals has a major influence on the magnitude and specificity of the CTL response against influenza A virus. Even in individuals who share a certain HLA allele, the CTL response restricted by this HLA allele may be affected by other nonmatching HLA molecules resulting in different CTL responses. This finding may have implications on vaccination strategies aiming at the induction of CD8+ T cell responses.

References

1. **Badovinac, V. P., A. R. Tvinnereim, and J. T. Harty.** 2000. Regulation of antigen-specific CD8(+) T cell homeostasis by perforin and interferon-gamma. Science. **290:**1354-8.
2. **Bednarek, M. A., S. Y. Sauma, M. C. Gammon, G. Porter, S. Tamhankar, A. R. Williamson, and H. J. Zweerink.** 1991. The minimum peptide epitope from the influenza virus matrix protein. Extra and intracellular loading of HLA-A2. J. Immunol. **147:**4047-53.
3. **Belz, G. T., J. D. Altman, and P. C. Doherty.** 1998. Characteristics of virus-specific CD8(+) T cells in the liver during the control and resolution phases of influenza pneumonia. Proc. Natl. Acad. Sci. U. S. A. **95:**13812-7.
4. **Belz, G. T., P. G. Stevenson, and P. C. Doherty.** 2000. Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. J. Immunol. **165:**2404-9.
5. **Belz, G. T., W. Xie, J. D. Altman, and P. C. Doherty.** 2000. A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T cell response is much less apparent following secondary challenge. J. Virol. **74:**3486-93.

6. **Belz, G. T., W. Xie, and P. C. Doherty.** 2001. Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. J. Immunol. **166:**4627-33.
7. **Bennink, J. R., and J. W. Yewdell.** 1988. Murine cytotoxic T lymphocyte recognition of individual influenza virus proteins. High frequency of nonresponder MHC class I alleles. J. Exp. Med. **168:**1935-9.
8. **Berzofsky, J. A.** 1988. Immunodominance in T lymphocyte recognition. Immunol. Lett. **18:**83-92.
9. **Betts, M. R., J. P. Casazza, B. A. Patterson, S. Waldrop, W. Trigona, T. M. Fu, F. Kern, L. J. Picker, and R. A. Koup.** 2000. Putative immunodominant human immunodeficiency virus-specific CD8(+) T cell responses cannot be predicted by major histocompatibility complex class I haplotype. J. Virol. **74:**9144-51.
10. **Blattman, J. N., D. J. D. Sourdive, K. Murali-Krishna, R. Ahmed, and J. D. Altman.** 2000. Evolution of the T Cell Repertoire During Primary, Memory, and Recall Responses to Viral Infection. J. Immunol. **165:**6081-6090.
11. **Bousso, P., F. Lemaitre, J. Bilsborough, and P. Kourilsky.** 2000. Facing two T cell epitopes: a degree of randomness in the primary response is lost upon secondary immunization. J. Immunol. **165:**760-7.
12. **Chen, W., S. Khilko, J. Fecondo, D. H. Margulies, and J. McCluskey.** 1994. Determinant selection of major histocompatibility complex class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by nondominant anchor residues. J. Exp. Med. **180:**1471-83.
13. **Chen, W., C. C. Norbury, Y. Cho, J. W. Yewdell, and J. R. Bennink.** 2001. Immunoproteasomes shape immunodominance hierarchies of antiviral cd8(+) t cells at the levels of t cell repertoire and presentation of viral antigens. J. Exp. Med. **193:**1319-26.
14. **Chen, W., J. W. Yewdell, R. L. Levine, and J. R. Bennink.** 1999. Modification of cysteine residues in vitro and in vivo affects the immunogenicity and antigenicity of major histocompatibility complex class I-restricted viral determinants. J. Exp. Med. **189:**1757-64.
15. **Crotzer, V. L., R. E. Christian, J. M. Brooks, J. Shabanowitz, R. E. Settlage, J. A. Marto, F. M. White, A. B. Rickinson, D. F. Hunt, and V. H. Engelhard.** 2000. Immunodominance among EBV-derived epitopes restricted by HLA-B27 does not correlate with epitope abundance in EBV-transformed B-lymphoblastoid cell lines. J. Immunol. **164:**6120-9.
16. **Daly, K., P. Nguyen, D. L. Woodland, and M. A. Blackman.** 1995. Immunodominance of major histocompatibility complex class I-restricted influenza virus epitopes can be influenced by the T cell receptor repertoire. J. Virol. **69:**7416-22.
17. **DiBrino, M., T. Tsuchida, R. V. Turner, K. C. Parker, J. E. Coligan, and W. E. Biddison.** 1993. HLA-A1 and HLA-A3 T cell epitopes derived from influenza virus proteins predicted from peptide binding motifs. J. Immunol. **151:**5930-5.

18. **Doherty, P. C., W. E. Biddison, J. R. Bennink, and B. B. Knowles.** 1978. Cytotoxic T cell responses in mice infected with influenza and vaccinia viruses vary in magnitude with H-2 genotype. J. Exp. Med. **148:**534-43.
19. **Dong, T., D. Boyd, W. Rosenberg, N. Alp, M. Takiguchi, A. McMichael, and S. Rowland-Jones.** 1996. An HLA-B35-restricted epitope modified at an anchor residue results in an antagonist peptide. Eur. J. Immunol. **26:**335-9.
20. **Flores-Villanueva, P. O., E. J. Yunis, J. C. Delgado, E. Vittinghoff, S. Buchbinder, J. Y. Leung, A. M. Uglialoro, O. P. Clavijo, E. S. Rosenberg, S. A. Kalams, J. D. Braun, S. L. Boswell, B. D. Walker, and A. E. Goldfeld.** 2001. Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. Proc. Natl. Acad. Sci. U. S. A. **98:**5140-5.
21. **Gallimore, A., T. Dumrese, H. Hengartner, R. M. Zinkernagel, and H. G. Rammensee.** 1998. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. J. Exp. Med. **187:**1647-57.
22. **Gallimore, A., J. Hombach, T. Dumrese, H. G. Rammensee, R. M. Zinkernagel, and H. Hengartner.** 1998. A protective cytotoxic T cell response to a subdominant epitope is influenced by the stability of the MHC class I/peptide complex and the overall spectrum of viral peptides generated within infected cells. Eur. J. Immunol. **28:**3301-11.
23. **Gavioli, R., M. G. Kurilla, P. O. de Campos-Lima, L. E. Wallace, R. Dolcetti, R. J. Murray, A. B. Rickinson, and M. G. Masucci.** 1993. Multiple HLA A11-restricted cytotoxic T lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. J. Virol. **67:**1572-8.
24. **Gianfrani, C., C. Oseroff, J. Sidney, R. W. Chesnut, and A. Sette.** 2000. Human memory CTL response specific for influenza A virus is broad and multispecific. Hum. Immunol. **61:**438-52.
25. **Gotch, F., A. McMichael, G. Smith, and B. Moss.** 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. J. Exp. Med. **165:**408-16.
26. **Hill, A. V., J. Elvin, A. C. Willis, M. Aidoo, C. E. Allsopp, F. M. Gotch, X. M. Gao, M. Takiguchi, B. M. Greenwood, A. R. Townsend, and et al.** 1992. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. Nature. **360:**434-9.
27. **Huet, S., D. F. Nixon, J. B. Rothbard, A. Townsend, S. A. Ellis, and A. J. McMichael.** 1990. Structural homologies between two HLA B27-restricted peptides suggest residues important for interaction with HLA B27. Int. Immunol. **2:**311-6.
28. **Jameson, J., J. Cruz, and F. A. Ennis.** 1998. Human cytotoxic T lymphocyte repertoire to influenza A viruses. J. Virol. **72:**8682-9.
29. **Jameson, J., J. Cruz, M. Terajima, and F. A. Ennis.** 1999. Human CD8+ and CD4+ T lymphocyte memory to influenza A viruses of swine and avian species. J. Immunol. **162:**7578-83.
30. **Jeffery, K. J., A. A. Siddiqui, M. Bunce, A. L. Lloyd, A. M. Vine, A. D. Witkover, S. Izumo, K. Usuku, K. I. Welsh, M. Osame, and C. R. Bangham.**

2000. The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection. J. Immunol. **165:**7278-84.
31. **Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. Hill, and A. J. McMichael.** 1997. Rapid effector function in CD8+ memory T cells. J. Exp. Med. **186:**859-65.
32. **Lukacher, A. E., V. L. Braciale, and T. J. Braciale.** 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. J. Exp. Med. **160:**814-26.
33. **Man, S., M. H. Newberg, V. L. Crotzer, C. J. Luckey, N. S. Williams, Y. Chen, E. L. Huczko, J. P. Ridge, and V. H. Engelhard.** 1995. Definition of a human T cell epitope from influenza A non-structural protein 1 using HLA-A2.1 transgenic mice. Int. Immunol. **7:**597-605.
34. **Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador.** 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature. **409:**1055-60.
35. **Martinon, F., E. Gomard, C. Hannoun, and J. P. Levy.** 1990. In vitro human cytotoxic T cell responses against influenza A virus can be induced and selected by synthetic peptides. Eur. J. Immunol. **20:**2171-6.
36. **Masurel, N., P. Ophof, and P. de Jong.** 1981. Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. J. Hyg. (Lond). **87:**201-9.
37. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare.** 1983. Cytotoxic T cell immunity to influenza. N. Engl. J. Med. **309:**13-7.
38. **Mo, A. X., S. F. van Lelyveld, A. Craiu, and K. L. Rock.** 2000. Sequences that flank subdominant and cryptic epitopes influence the proteolytic generation of MHC class I-presented peptides. J. Immunol. **164:**4003-10.
39. **Morrison, J., J. Elvin, F. Latron, F. Gotch, R. Moots, J. L. Strominger, and A. McMichael.** 1992. Identification of the nonamer peptide from influenza A matrix protein and the role of pockets of HLA-A2 in its recognition by cytotoxic T lymphocytes. Eur. J. Immunol. **22:**903-7.
40. **Mullbacher, A., M. Lobigs, J. W. Yewdell, J. R. Bennink, R. Tha Hla, and R. V. Blanden.** 1999. High peptide affinity for MHC class I does not correlate with immunodominance. Scand. J. Immunol. **50:**420-6.
41. **Palmer, D., W. Dowdle, M. Coleman, and G. Schild** 1975. Haemagglutination inhibition test, p. 25-62. *In* Anonymous (ed.), Advanced laboratory techniques for influenza diagnosis. Procedural Guide. US Dept. Hlth. ED., Atlanta.
42. **Rimmelzwaan, G. F., M. Baars, E. C. Claas, and A. D. Osterhaus.** 1998. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J Virol Methods. **74:**57-66.
43. **Rimmelzwaan, G. F., N. Nieuwkoop, A. Brandenburg, G. Sutter, W. E. Beyer, D. Maher, J. Bates, and A. D. Osterhaus.** 2000. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune

responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine. **19:**1180-7.
44. **Robbins, P. A., P. A. Rota, and S. Z. Shapiro.** 1997. A broad cytotoxic T lymphocyte response to influenza type B virus presented by multiple HLA molecules. Int. Immunol. **9:**815-23.
45. **Rowland-Jones, S. L., T. Dong, K. R. Fowke, J. Kimani, P. Krausa, H. Newell, T. Blanchard, K. Ariyoshi, J. Oyugi, E. Ngugi, J. Bwayo, K. S. MacDonald, A. J. McMichael, and F. A. Plummer.** 1998. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. J. Clin. Invest. **102:**1758-65.
46. **Silins, S. L., S. M. Cross, S. L. Elliott, S. J. Pye, S. R. Burrows, J. M. Burrows, D. J. Moss, V. P. Argaet, and I. S. Misko.** 1996. Development of Epstein-Barr virus-specific memory T cell receptor clonotypes in acute infectious mononucleosis. J. Exp. Med. **184:**1815-24.
47. **Sutton, J., S. Rowland-Jones, W. Rosenberg, D. Nixon, F. Gotch, X. M. Gao, N. Murray, A. Spoonas, P. Driscoll, M. Smith, and et al.** 1993. A sequence pattern for peptides presented to cytotoxic T lymphocytes by HLA B8 revealed by analysis of epitopes and eluted peptides. Eur. J. Immunol. **23:**447-53.
48. **Tussey, L. G., S. Rowland-Jones, T. S. Zheng, M. J. Androlewicz, P. Cresswell, J. A. Frelinger, and A. J. McMichael.** 1995. Different MHC class I alleles compete for presentation of overlapping viral epitopes. Immunity. **3:**65-77.
49. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, and et al.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. **259:**1745-9.
50. **van der Most, R. G., A. Sette, C. Oseroff, J. Alexander, K. Murali-Krishna, L. L. Lau, S. Southwood, J. Sidney, R. W. Chesnut, M. Matloubian, and R. Ahmed.** 1996. Analysis of cytotoxic T cell responses to dominant and subdominant epitopes during acute and chronic lymphocytic choriomeningitis virus infection. J. Immunol. **157:**5543-54.
51. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J. Virol. **74:**6800-7.
52. **Yewdell, J. W., and J. R. Bennink.** 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu. Rev. Immunol. **17:**51-88.

Chapter 3

Preferred HLA usage and HLA dependent differential cytokine expression in the influenza virus-specific cytotoxic T lymphocyte response

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Submitted for publication

Abstract

In order to study whether individual HLA class I alleles are used preferentially or equally in human virus-specific CTL responses, the contribution of individual HLA-A and –B alleles to the human influenza virus-specific CTL response was investigated. To this end, PBMC were obtained from three groups of HLA-A and –B identical blood donors and stimulated with influenza virus. In the virus-specific CD8+ T cell population the proportion of IFN-γ and TNF-α producing cells, restricted by individual HLA-A and -B alleles was determined using virus-infected C1R cells expressing a single HLA-A or –B allele for restimulation of these cells. In HLA-B*2705- and HLA-B*3501-positive individuals, these alleles were preferentially used in the influenza A virus-specific CTL response, while the contribution of HLA-B*0801 and HLA-A*0101 was minor in these donors. The magnitude of the HLA-B*0801-restricted response was even lower in the presence of HLA-B*2705 indicating that the CTL response restricted by a single HLA molecule is influenced by the coexpression of another. C1R cells expressing HLA-B*2705, HLA-A*0101 or HLA-A*0201 were preferentially lysed by virus-specific CD8+ T cells. In contrast, the CTL response to influenza B virus was mainly directed towards HLA-B*0801-restricted epitopes. Thus, the preferential use of HLA alleles depended on the virus studied. Finally, analysis of IFN-γ and TNF-α expression demonstrated that HLA-A*0101-restricted CD8+ T cells produce relatively more TNF-α than IFN-γ, in comparison to CD8+ T cells restricted by other alleles. Thus, differential cytokine expression profiles were identified in influenza virus-specific CTL depending on the epitopes recognized and/or their restriction elements.

Introduction

Cytotoxic T lymphocytes (CTL) have been shown to play an important role in the control and clearance of virus infections including those caused by influenza virus (4, 19). Although a large number of peptides are generated during processing of viral proteins in infected cells, only some of these peptides are ultimately presented by major histocompatibility complex (MHC) class I molecules and recognized by specific CTL. This limited recognition of a small number of dominant CTL epitopes has been termed immunodominance (3, 5, 33). Usually immunodominance is defined using a number of synthetic peptides representing known CTL epitopes, without taking into account the full repertoire of potential epitopes. Little information is available on the complete repertoire of CTL epitopes of viral pathogens and the corresponding human leukocyte antigen (HLA) alleles presenting these epitopes to specific CTL. Using transient HLA expression systems, HLA-A11, HLA-B8 and HLA-B44 were identified as the preferred restriction elements in the CTL response to Epstein-Barr virus (EBV) (15, 17, 22, 27). These studies also demonstrated that the HLA-A1-restricted EBV-specific CTL response is of minor importance.

In most studies addressing the contribution of individual HLA molecules in CTL responses, synthetic peptides representing known CTL epitopes have been used (6, 7, 11, 13, 20, 28). It has been shown recently that the magnitude and specificity of influenza A virus-specific CTL response, using nine peptide analogues of known influenza A virus CTL epitopes, is related to the MHC class I haplotype of individuals (7). In this study it was demonstrated that the magnitude of the influenza A virus-specific CTL response was highest in HLA-A*0201-positive individuals. This association was thought to be related to recognition of an HLA-A*0201-restricted immunodominant epitope ($M1_{58-66}$) from the matrix protein. Thus it might be expected that if CTL responses were analyzed by individual HLA-A and -B alleles, the majority of CTL would recognize influenza A virus-infected targets in a HLA-A*0201-restricted fashion. Although the use of synthetic peptides provide some information on HLA usage in CTL response, this approach has limitations and does not account for the full repertoire of viral epitopes presented by infected cells.

In the present study, the contribution of individual HLA-A and -B alleles to the CTL response against a model viral pathogen was investigated. To this end, the influenza virus-specific memory CTL response was studied, using C1R cells expressing a single HLA-A or -B allele and influenza virus-stimulated PBMC obtained from three groups of healthy blood donors with defined HLA class I haplotypes. Between groups the individuals shared two or three of the four HLA-A and –B alleles. Intracellular gamma-interferon (IFN-γ) and tumor necrosis factor (TNF)-α staining was used for the enumeration of virus-specific CTL restricted by a single HLA-A or -B allele and the lytic capacity of these cells was determined in classical ^{51}Cr-release assays.

We found that individual HLA-alleles were not used to the same extent and that in each group of HLA-typed individuals a hierarchy existed between HLA-alleles. Especially HLA-B*2705 and HLA-B*3501 were found to be preferred alleles in the influenza A virus-specific CTL response. The preferred HLA usage was found to

depend on the type of influenza virus (A or B) studied. In addition, for some alleles (e.g. HLA-A*0101) a discrepancy was observed between IFN-γ and TNF-α production indicating that CTL exhibited functional diversity depending on the epitopes recognized and/or their HLA class I restriction element.

Material and Methods

Cells

Peripheral blood mononuclear cells (PBMC) obtained from 13 healthy blood donors (Tab. 1) were isolated between 1999 and 2002 using Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and cryopreserved at -135°C. Three groups of donors, aged between 35 and 50 years of age, were selected according to serological homology within the A locus and B locus of HLA class I molecules (Tab. 1). Genetic subtyping was performed in the laboratory for Histocompatibility and Immunogenetics at the Bloodbank Rotterdam (Rotterdam, Netherlands) using a commercial typing system (GenoVision, Vienna, Austria). All donors had serum antibodies against one or more influenza A virus strains, measured by hemagglutination inhibition assay (HAI), indicative of one or more exposures in the past. Also three of the four donors tested for influenza B virus-specific CTL immunity (group II), had influenza B virus-specific antibodies.

Table 1: HLA-A- and –B genotype of donors

Group	HLA-A and –B genotype	No. of donors
I	A*0101, A*0201, B*0801, B*3501	4
II	A*0101, A*0201, B*0801, B*2705	4
III	A*0101, A*0301, B*0801, B*3501	5

Of each group of HLA-A- and –B-matched donors, EBV-transformed B-lymphoblastoid cell-lines (BLCL) were produced as described previously (24). BLCL were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamin, 100 μg/ml streptomycin and 100 IU/ml penicillin (R10F).

Hmy2-C1R (C1R) is a human BLCL with low expression of endogenous HLA-Cw4 (34, 35), but no expression of HLA-A or –B alleles. Control C1R cells and those transfected with individual HLA-A or –B genes were cultured in R10F. The transfected C1R cells were kindly provided by several investigators; HLA-A1-transfected C1R cell-line by Dr. P. Cresswell (Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, USA), HLA-A2.1-transfected C1R cell-line by Dr. P. Romero (Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Lausanne, Switzerland), HLA-

A3-transfected C1R cell-line by Dr. W. Biddison (Molecular Immunology Section, National Institute of Neurological Disorders and Stroke, National Institute of Health, Bethesda, USA), HLA-B*2705-transfected C1R cell-line by Dr. J. Lopez de Castro (Centro de Biologia Molecular Severo Ochoa, Universidad Autonoma de Madrid, Facultad de Ciencias, Madrid, Spain) and HLA-B*3501-transfected C1R cell-line by Dr. M. Takiguchi (Division of Viral Immunology, Kumamoto University, Kumamoto, Japan). Finally, an HLA-B*0801-transfected C1R cell-line was established as described previously (paper submitted for publication). Although the genotype of the majority of these HLA-transfected C1R cell-lines is known, the cell-lines will be depicted by their serotype throughout the manuscript (for example C1R-B27). Because C1R-A1 cells gradually loose the expression of HLA-A1, an enrichment procedure was performed every two to three weeks using anti-HLA-A1, 36 monoclonal antibodies (MAb, BIH0331, One Lambda Inc., Canoga Park, CA, USA) together with DNA-linked anti-mouse-Ig beads (Dynal Biotech GmBH, Hamburg, Germany) according to manufacturers instructions, to ensure a high percentage of HLA-A1+ C1R cells (minimal of 80% HLA-A1+ cells).

Serology
Plasma samples were stored at –20°C and tested for the presence of influenza A virus (H3N2) and influenza B virus-specific antibodies in an HAI assay according to standard methods (18, 21) using turkey erythrocytes and four hemagglutinating units of virus. The sera were tested for antibodies against eleven vaccine strains of subtype H3N2 used since 1968 and eleven influenza B virus strains (from 1979 till 2001). Ferret sera raised against the test antigens were used as positive controls.

Influenza viruses and peptides
Sucrose gradient purified influenza viruses Resvir-9 and B/Harbin/7/94 (kindly provided by CSL Ltd., Parkville, Victoria, Australia) were used for infection of cells. Resvir-9 is a reassortant virus between strains A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2), containing the nucleoprotein (NP), hemagglutinin (HA) and neuraminidase (NA) of A/Nanchang/933/95. The infectious virus titers of the virus preparations were determined in cell-culture using Madin-Darby-Canine-Kidney (MDCK) cells as indicator cells, as described previously (23). Peptide analogs of influenza A virus CTL epitopes were manufactured, HPLC purified and analyzed by mass spectrometry (Eurogentec, Seraing, Belgium). The peptide analogs correspond to the HLA-A1-restricted NP_{44-52}, HLA-A*0201-restricted $M1_{58-66}$, HLA-A3-restricted $NP_{265-273}$, HLA-B*0801-restricted $NP_{380-388}$, HLA-B*2705-restricted $NP_{383-391}$ and HLA-B*3501-restricted $NP_{418-426}$ epitope. The peptides were dissolved in DMSO at 5.0 mg/ml, diluted to 100 μM in RPMI 1640 and stored at -20°C.

In vitro stimulation of PBMC with influenza virus
Stimulation of PBMC with influenza virus was performed as described previously (7). Cells were resuspended at $1x10^6$ cells/ml in R10F and infected for one hour at 37°C with Resvir-9 or B/Harbin/7/94, at a multiplicity of infection (MOI) of three.

Next, the cells were washed once and resuspended in RPMI 1640 medium supplemented with 10% human AB serum, 2 mM glutamin, 100 μg/ml streptomycin, 100 IU/ml penicillin and 20 μM ß-mercaptoethanol (R10H) and added to uninfected PBMC at a ratio of 1:1 in a 25 cm^2 culture flask. After two days, recombinant interleukin-2 (rIL-2, final concentration 50 U/ml, Chirion B.V., Amsterdam, Netherlands) was added and the cells were incubated for another six days at 37°C and used as effector cells in a ^{51}Cr-release assay or intracellular cytokine staining (ICS) assay (see below).

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). First the cells were washed once in PBS supplemented with 2.0% FCS (P2F) and finally resuspended in P2F at a concentration of $1x10^7$/ml. Capture beads were added to the cell suspension at a bead to CD8+ T cell ratio of 8:1. Following a 30-minute incubation at 4°C, the beads/cells were washed six times with 5.0 ml of P2F. The beads, together with the attached cells, were reconstituted in 200 μl of RPMI 1640 medium with 1.0% FCS. To detach the cells from the beads, 20 μl of DETACHaBEAD® (Dynal Biotech GmBH) was added and incubated for one hour at 20°C. The released cells were isolated, washed once in R10F and used as effector cells in ^{51}Cr-release assays (16).

Preparation of target cells for ^{51}Cr-release assay
HLA-A- and –B-matched BLCL, C1R and C1R cells transfected with various HLA genes were used as target cells in ^{51}Cr-release assays. All cells were infected with Resvir-9 at a MOI of three in RPMI 1640 medium, containing 0.1% bovine serum albumin, 2 mM glutamin, 100 μg/ml streptomycin and 100 IU/ml penicillin (R0.1B). Following a one-hour infection at 37°C, the cells were washed once in R10F and incubated in R10F for 16 h at 37°C. The following day $1x10^6$ cells were washed once in R0.1B medium and incubated for one hour at 37°C with 75μCi $Na_2[^{51}Cr]O_4$. The cells were then washed three times in R10F and used as target cells. Uninfected cells of the HLA-matched BLCL, C1R and HLA-transfected C1R cell-line were included to determine non-specific lysis of target cells.
The antigen presentation capacity of HLA-transfected C1R cells was compared with cells of two BLCL. To this end, the minimal peptide concentration was determined for which 50% of the target cells were killed (EC_{50}) by epitope-specific CTL clones. After a one-hour incubation with $Na_2[^{51}Cr]O_4$, the cells were washed twice in R10F and distributed in 96-well V-bottom plates. Next, the cells were incubated with a ten-fold serial dilution of the peptides in R10F for one hour at 37°C, washed once in R10F and used as target cells in a ^{51}Cr-release assay. The HLA-A1-, -A2-, -A3-, -B8-, -B27-, and -B35-restricted influenza virus-specific CTL clones were previously described (8, 31, 32).

Intracellular cytokine staining
Influenza virus-stimulated PBMC were also used for ICS assay. One hundred thousand PBMC were incubated in 100 µl R10F containing GolgiStop (monensin, [Pharmingen, Alphen a/d Rijn, Netherlands]) and GolgiPlug (brefeldin, [Pharmingen]). In addition, $2x10^5$ influenza virus-infected (Resvir-9, MOI = 3 or B/Harbin/7/94, MOI = 1) and uninfected HLA-matched BLCL cells and C1R cells with or without HLA transgene were also incubated in 100 µl R10F containing GolgiPlug and GolgiStop and used as stimulator cells. After 30 minutes at 37°C, the stimulator cells were added to the PBMC for six hours at 37°C. Next the cells were washed, stained and fixed as previously described (10) using anti-CD8 (Dako, Glostrup, Denmark), anti-CD3 (Becton Dickinson, Alphen a/d Rijn, Netherlands),

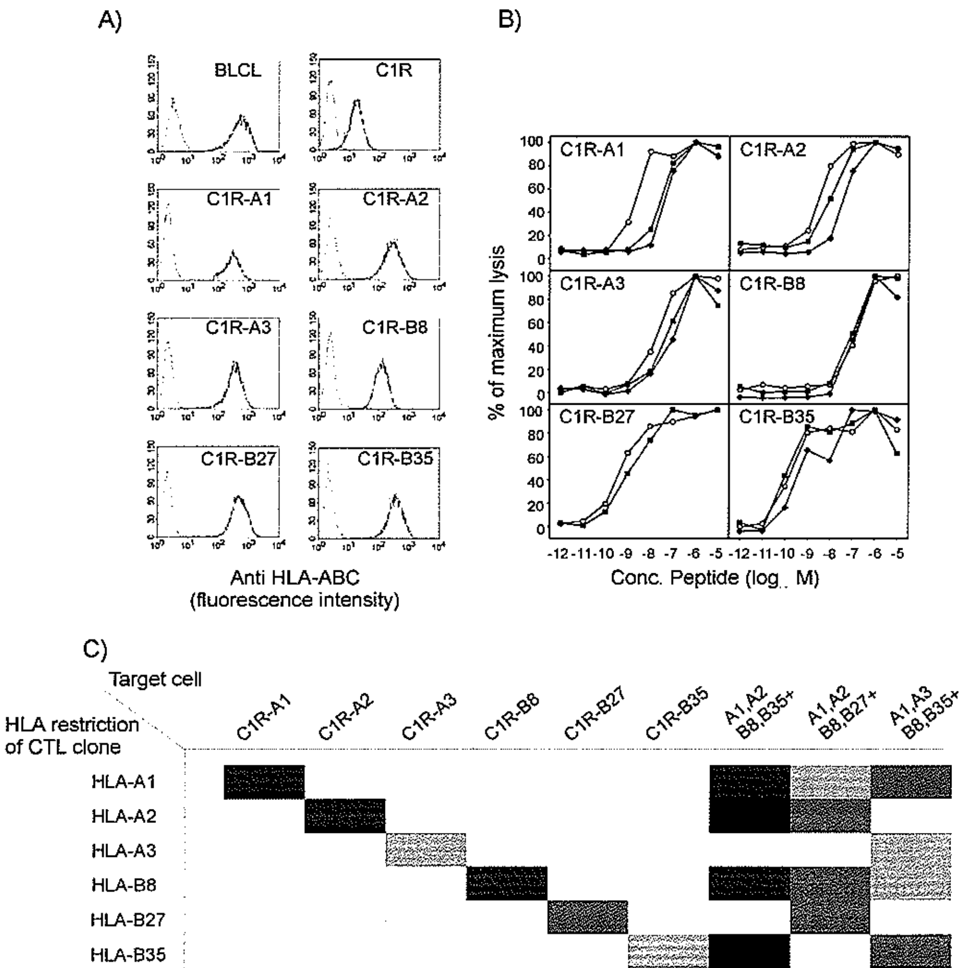

Figure 1: Expression of foreign HLA-A or –B alleles on C1R cells.
(A) HLA expression on BLCL, C1R and HLA-transfected C1R cells. HLA expression was measured with saturating levels of anti-HLA-ABC MAb. The level of HLA expression (fluorescence intensity, solid line) is shown for cells of BLCL, C1R and HLA-transfected C1R cell-lines. Unstained cells are shown as dotted lines in the figures.
(B) HLA-transfected C1R cell-lines stimulate influenza A virus-specific CTL clones. HLA-transfected C1R cells (open symbols) and two HLA-matched BLCL (closed symbols) were used as target cells in a ^{51}Cr-release assay after a one-hour incubation with 10-fold serial dilutions of peptide analogous of epitopes. C1R-A1 cells were incubated with the NP_{44-52} epitope, C1R-A2 cells with the $M1_{58-66}$ epitope, C1R-A3 cells with the $NP_{265-273}$ epitope, C1R-B8 cells with the $NP_{380-388}$ epitope, C1R-B27 cells with $NP_{383-391}$ epitope and finally C1R-B35 cells with the $NP_{418-426}$ epitope and used as target cells for CTL clones specific for the respective epitopes (effector-to-target cell (E:T) ratio of 10:1).
(C) HLA-restricted recognition of influenza A virus-infected HLA-transfected C1R cells by virus-specific CTL. HLA-transfected C1R cells, C1R control cells and three different BLCL cells were infected with Resvir-9 (MOI = 3) and used as target cells in a ^{51}Cr-release assay. Influenza A virus-specific CTL clones (see above) were used as effector cells at an E:T ratio of 10:1. The results are given as the % lysis of each target cell minus the lysis of virus-infected C1R cells. HLA-genotyped BLCL were included as controls to confirm the HLA restriction of the influenza A virus-specific CTL clones.
The percentage lysis is represented by different colors (▇, 100%-80%; ▇, 80%-60%; ▇, 60%-40%; ▇, 40%-20%, ☐, 20%-0%).

anti-IFN-γ (Pharmingen) and anti-TNF-α (Becton Dickinson) MAb. At least 2000 gated CD3+ CD8+ T cells were acquired using a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed with CellQuest Pro (Becton Dickinson) and are expressed as the % cytokine-positive cells following stimulation with influenza virus-infected cells minus the % cytokine-positive cells after stimulation with uninfected cells. To determine the % allele-specific cytokine-positive T cells, the proportion of cytokine-positive cells following stimulation with C1R control cells is subtracted from the % cytokine-positive cells following stimulation with HLA-transfected C1R cells.

^{51}Cr-release assay

^{51}Cr-release assays were performed as described previously (7). Influenza A virus-specific CTL clones were added to 5 x 10^3 ^{51}Cr-labeled target cells at an effector-to-target cell ratio (E:T ratio) of 10:1 or 5:1. Isolated CD8+ T cells, obtained from influenza A virus stimulated PBMC cultures, were also used as effector cells at E:T ratios of 10:1 to 1.25:1. After four hours at 37°C, the culture supernatants were harvested (Skatron instruments, Sterling, Va., USA) and radioactivity was measured by gamma-counting. The % specific lysis was calculated with the following formula: [(experimental release - spontaneous release) / (maximum release - spontaneous release)] x 100. The % influenza A virus-specific lysis was calculated from the % lysis of infected cells minus the percentage lysis of uninfected cells of at least three wells.

Flow cytometry

Following isolation of CD8+ T cells the purity of the CD8+ T cell population was tested. Approximately $1x10^5$ cells were washed once in P2F, and incubated in 50 μl P2F containing anti-CD8, anti-CD4 (Dako) and anti-CD3 MAb for 30 minutes at 4°C. Next the cells were washed once in P2F and at least $1x10^4$ events were acquired using a FACSCalibur flow cytometer. The CD8+ T cell purity of the isolated CD8+ T cells was generally > 90%.

Prior to ^{51}Cr-release and ICS assays, target cells were tested for the expression of transfected HLA genes. C1R-A1 and C1R-A3 cells were incubated with biotin-conjugated anti-HLA-A1/36 and anti-HLA-A3 (BIH0269, One Lambda Inc.) MAb respectively, followed by FITC-conjugated streptavidin (Dako). C1R-A2, C1R-B8 and C1R-B27 cells were incubated with FITC-conjugated anti-HLA-A2/28 (FH0037, One Lambda Inc.), anti-HLA-B8 (FH0536A, One Lambda Inc.) or anti-HLA-B27 (B27F50X, One Lambda Inc.) MAb respectively. Finally C1R-B35 were incubated with culture supernatant of hybridoma 4D12 (ATCC No. HB-178, ATCC, Manansass, VA, USA), and subsequently stained with FITC-conjugated anti-mouse-Ig F_{ab} fragments (Dako). The level of HLA expression on C1R and C1R cells with various HLA transgenes was determined by flow cytometry using saturating levels of anti-HLA-ABC antibodies (Pharmingen). All the antibody staining procedures were performed in P2F at 4°C. At least 10000 cells were acquired with a FACSCalibur flow cytometer and analyzed with CellQuest Pro.

Statistical analysis

To identify statistical differences between IFN-γ responses restricted by individual HLA-A or –B alleles a student *t* test was performed. To investigate preferential HLA-A or –B usage in the three groups of donors, a Friedman test was performed, comparing the response to individual alleles with a theoretical random contribution of the four HLA-A and –B alleles. Differences in ratio IFN-γ+ cells and TNF-α+ cells were analyzed with a univariate ANOVA post-hoc analysis. Ratio IFN-γ/TNF-α positive cells was determined if both proportions cytokine-positive cells > 1.0%. *P*-values of < 0.05 were considered statistically significant.

Results

Validation of HLA-transfected C1R cells

Before using C1R cells expressing HLA transgenes as antigen presenting cells (APC), their HLA expression and antigen presenting capacity was tested. As shown in figure 1a, all C1R cells transgenic for individual HLA genes exhibited HLA class I expression after incubation with an antibody specific for all HLA-A, -B and -C alleles. The expression was in the same order of magnitude as in normal BLCL cells (solid lines, figure 1a). Control C1R cells also exhibited surface expression of HLA class I molecules to a limited extend as a result of endogenous HLA-Cw4 expression (34, 35). This expression was at least 10-fold lower than in the HLA-transfected C1R cells. The expression of HLA-A or –B molecules by the respective C1R cells was further confirmed using allele-specific antibody preparations (data not shown).

Furthermore, the HLA expression was not reduced after infection of these cells with influenza A viruses (data not shown).
The concentration of peptide analogous of CTL epitopes required to give 50% of the maximum lysis (EC_{50} values) of target cells by corresponding CTL clones was used as a measure for antigen presenting capacity of C1R cells. As shown in figure 1b, the EC_{50} values ranged from 10^{-7} M for the HLA-B*0801-restricted epitope ($NP_{380-388}$) to 10^{-10} M for the HLA-B*3501-restricted epitope ($NP_{418-426}$). More importantly the EC_{50} values for the HLA-transfected C1R cells were similar or slightly lower than the EC_{50} values measured with HLA-matched BLCL cells, indicating that functional presentation of peptides is comparable.

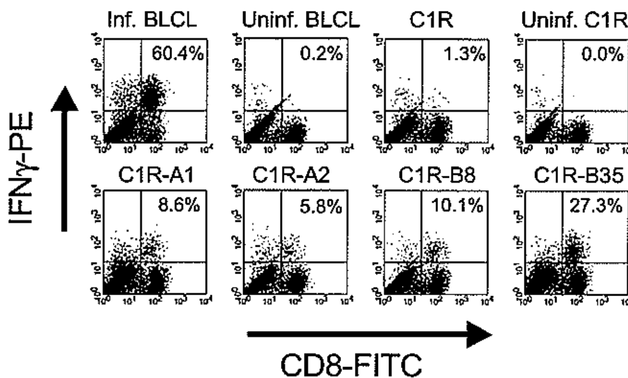

Figure 2: IFN-γ expression in CD8+ T cells after stimulation of PBMC with influenza virus.
PBMC expanded after stimulation with influenza A virus were restimulated with HLA-matched BLCL, C1R and HLA-transfected C1R cells infected with influenza A virus (Resvir-9) at a ratio of 1:2 for six hours in the presence of monensin and brefeldin. Stimulation with uninfected cells was used as negative controls. Virus-specific CTL restricted by individual alleles were visualized after staining with MAb specific for CD8, CD3 and IFN-γ. The dotplots show an example of the expression of IFN-γ in T cells following stimulation of PBMC of donor 1 (group I) with infected and uninfected cells of an HLA-matched BLCL and C1R control cells and infected C1R cells expressing HLA-A1, HLA-A2, HLA-B8 or HLA-B35. The proportion IFN-γ+ cells in the CD3+ CD8+ T cell fraction is indicated.

Using HLA-transfected C1R cells infected with influenza A virus it was demonstrated in a ^{51}Cr-release assay that the natural CTL epitope was liberated from viral proteins and presented to specific CTL clones (figure 1c). In addition, the respective CTL clones only recognized HLA-transfected C1R target cells expressing the matching HLA molecule.

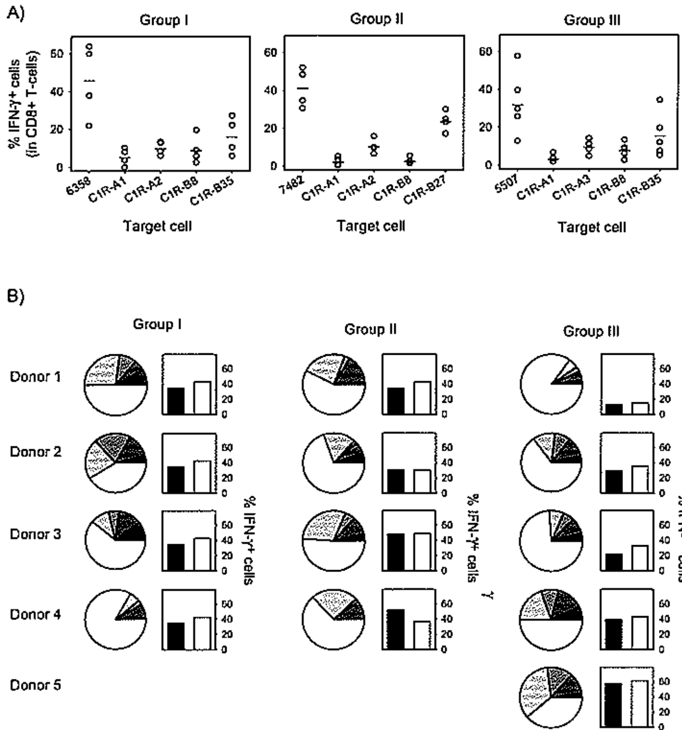

Figure 3: Influenza A virus-specific IFN-γ responses restricted by individual HLA-A and –B alleles in three groups of HLA-A and -B matched donors.

A) Virus-specific effector cells expanded from PBMC were restimulated with influenza A virus-infected and uninfected HLA-matched BLCL, C1R and HLA-transfected C1R cells at a ratio of 1:2 for six hours in the presence of monensin and brefeldin. Virus-specific CTL were visualized after staining for CD8, CD3 and IFN-γ. The proportion of IFN-γ+ cells in the CD3+ CD8+ T cell fraction after stimulation with infected HLA-matched BLCL cells (6358, 7482 and 5507) or C1R cells expressing individual HLA-A or –B alleles is shown. The proportion influenza A virus-specific IFN-γ+ CD8+ T cells was calculated by subtracting the % of IFN-γ+ CD8+ T cells following stimulation with uninfected cells and virus-infected C1R control cells from the % IFN-γ+ CD8+ T cells obtained after stimulation with virus-infected HLA-transfected C1R cells.

B) The contribution of HLA-A and –B alleles in the influenza A virus-specific CTL response. The different parts in the pie-charts represent the proportion of CD8+ T cells restricted by individual HLA alleles (shades of grey), starting with HLA-A*0101 (■) and going anti-clock wise via HLA-A*0201 in group I and II (▩) or HLA-A*0301 in group III (▩), and HLA-B*0801 (▩) to HLA-B*2705 in group II (░) and HLA-B*3501 in group III (░). The proportion of unresponsive CD8+ T cells is also shown (□). The bar-charts indicate the percentage influenza A virus-specific IFN-γ+ CD8+ T cells after stimulation with HLA-matched BLCL cells (black bars) or the sum of the percentage IFN-γ+ CD8+ T cells after stimulation with C1R cells transfected with individual HLA-A and –B alleles (white bars). The average proportion of IFN-γ+ CD8+ T cells of all donors in group I-III is shown in figure 3a by horizontal bars (---).

IFN-γ responses in CD8+ T cells restricted by individual HLA-A and –B alleles

The contribution of individual HLA-A and –B alleles to the influenza A virus-specific CTL response was determined by measuring the proportion of IFN-γ+ CD8+ T cells in influenza A virus-specific PBMC cultures, which were restimulated with HLA-matched BLCL or C1R cells expressing individual HLA-A and –B alleles. An example of such an analysis is shown in figure 2.

Using this procedure, PBMC of all (n = 13) donors were tested. In donors of group I (HLA-A1, HLA-A2, HLA-B8, HLA-B35), the proportion of CD8+ T cells producing IFN-γ after stimulation with HLA-matched BLCL cells was 46% (figure 3a). Stimulation with HLA-transfected C1R cells showed that on average 16.4% of these cells were restricted by HLA-B*3501. HLA-A*0201-, HLA-B*0801- and HLA-A*0101-restricted CD8+ T cells accounted for 10.2%, 9,2% and 5,5% on average respectively. None of these differences were statistically significant.

In donors of group II (HLA-A1, HLA-A2, HLA-B8, HLA-B27) on average 41% of the CD8+ T cells were IFN-γ+ after stimulation with HLA-matched BLCL cells. The majority of these cells were specific for CTL epitopes presented in the context of HLA-B*2705 with an average of 23,8% of IFN-γ+ CD8+ T cells in these donors (figure 3a). This HLA-B*2705-restricted response differed significantly with the percentages IFN-γ+ CD8+ T cells found after stimulation with C1R-A2 (10,5%, P = 0.008), C1R-A1 (2,4%, P = 0.002) and C1R-B8 (2,9%, P = 0.002). The proportion IFN-γ+ cells after stimulation with C1R-A2 (10,5%) also differed from the responses to C1R-A1 (P = 0.016) and C1R-B8 (P = 0.02).

In donors of group III (HLA-A1, HLA-A3, HLA-B8, HLA-B35), the proportion of CD8+ T cells producing IFN-γ after stimulation with HLA-matched BLCL cells was on average 32% (figure 3a), which was lower than the proportion influenza virus-specific CTL found in group I (46%) and II (41%). Most of the influenza A virus-specific CD8+ T cells were specific for CTL epitopes presented by HLA-B*3501 (15,9% IFN-γ+ cells) followed by those presented by C1R-A3 (10,0%), C1R-B8 (7,8%) and C1R-A1 (3,5%). Although the average % IFN-γ+ cells of HLA-A*0101-restricted CD8+ T cells was lower than the proportion of cells restricted by the other alleles in this group, only the difference between the proportion of HLA-A*0101- and HLA-A*0301-restricted CTL were statistically significant (P = 0.007).

We also compared the magnitude of the responses restricted by shared alleles between donors of different groups. The HLA-B*0801-restricted response was lower in donors of group II (2.9%) than in those of group I (9.1%, P = 0.09) and group III (7.8%, P = 0.04). Also the HLA-A*0101-restricted response was slightly higher in group I (5.5%) than in group II (2.4%) and III (3.5%), although these differences were not statistically significant. No differences were found between groups in the proportion of IFN-γ+ CD8+ T cells restricted by HLA-A*0201 (10.2% vs. 10.5%) or HLA-B*3501 (16.4% vs. 15.9%).

To demonstrate preferential HLA usage in individual donors, pie charts were constructed (figure 3b), illustrating the IFN-γ responses for each HLA allele (shades of gray) within the CD8+ T cell population (entire circle). Not all CD8+ T cells

produced IFN-γ upon stimulation with virus-infected stimulator cells (white part). In donors of group I, an HLA-A*0201-restricted response was always found, which was dominant in donor 1 and 2. HLA-B*3501 was found to be an immunodominant allele in two other donors (3 and 4). Subdominant HLA-B*0801-restricted responses were found in all donors, while the HLA-A*0101-restricted response was detected in 3 out of 4 donors. Preferential HLA usage in this group of donors was not demonstrated ($P = 0.165$).

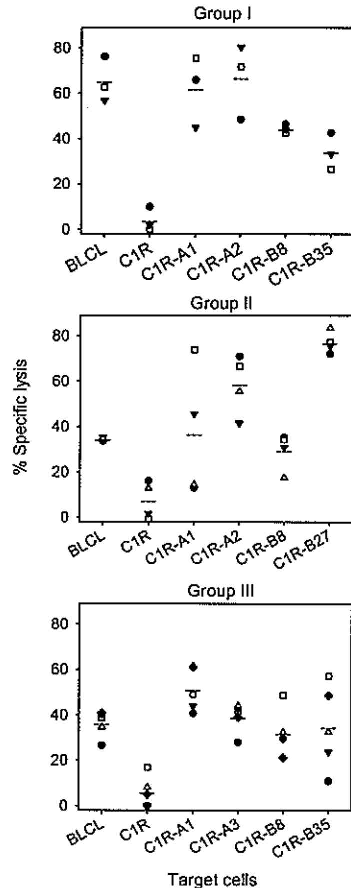

Figure 4: Lytic activity of CTL restricted by individual HLA-A and –B alleles.
CD8+ cells were isolated from influenza A virus stimulated PBMC and used as effector cells in a ^{51}Cr-release assay. The lytic activity of virus-specific CTL is calculated as the percentage lysis of influenza A virus-infected HLA-matched BLCL, C1R and HLA-transfected C1R cells minus the lysis of uninfected cells, at an effector to target cell ratio of 5:1 for HLA-matched BLCL cells and 10:1 for C1R and HLA-transfected C1R cells. Each graph represents a group of HLA-A and -B identical donors and each donor is depicted by a different symbol. Donor 4 in group I was excluded because insufficient numbers of cells were recovered to perform the assay. The average percentage lysis of target cells by effector cells of the respective donors is shown (---).

In all four donors of group II a dominant HLA-B*2705-restricted IFN-γ response was observed in addition to the HLA-A*0201-restricted IFN-γ response. The HLA-A*0101- and HLA-B*0801-restricted IFN-γ responses only contributed to a limited extend to the influenza A virus-specific CTL response in these donors. The preferred usage of certain HLA class I alleles in these donors was statistically significant ($P = 0.011$).
In group III, the most dominant response was restricted by HLA-B*3501 in donors 1, 2, 4 and 5, while in donor 3 this response was only minor (figure 3b). An HLA-

A*0301-restricted response was observed in all five donors and was found immunodominant in three donors. Also the HLA-A*0101- and B*0801-restricted responses were detected in all donors, but were found to be subdominant. Again the preferred recognition of certain alleles in these donors was statistically significant ($P = 0.005$).

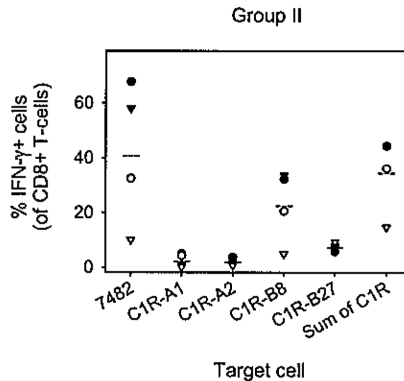

Figure 5: Influenza B virus-specific IFN-γ responses restricted by individual HLA-A and –B alleles in donors of group II.
A) Effector cells were restimulated with influenza B virus-infected (MOI=1) and uninfected HLA-matched BLCL cells, C1R cells and HLA-transfected C1R cells at a ratio of 1:2 for six hours in the presence of monensin and brefeldin. Influenza B virus-specific CTL were visualized after staining for CD8, CD3 and IFN-γ. The data represents the proportion of influenza B virus-specific IFN-γ+ cells in the CD3+ CD8+ T cell fraction after stimulation with HLA-matched BLCL cells (7482) and C1R cells expressing individual HLA alleles as indicated. In addition, the sum of the proportion IFN-γ+ CD8+ T cells restricted by individual HLA-A and –B alleles is depicted for each individual. The average proportion of IFN-γ+ CD8+ T cells of all donors in group II is shown by a horizontal bar (---). The symbols represent the four different donors from this group.

The sum of the IFN-γ+ CD8+ T cells observed after stimulation with four different C1R cell-lines expressing the four HLA-A and –B alleles, was compared with the number of IFN-γ+ CD8+ T cells observed after stimulation with HLA-matched BLCL cells. As shown in figure 3b (bar charts), the sum of the responses restricted by individual alleles (white bars) almost accounted for the total influenza A virus-specific response (black bars) in most cases. In 11 out of 13 donors the difference was less than 10% and only in donor 4 of group II and donor 3 of group III the differences exceeded 10%.

HLA-A- and -B-restricted lysis of target cells by CD8+ T cells

The contribution of individual HLA-A and -B alleles in the recognition of infected target cells by influenza A virus-specific CTL was also studied in ^{51}Cr-release

assays, using infected and uninfected HLA-transfected C1R cells and HLA-matched BLCL cells (figure 4). The CD8+ effector cell populations obtained from donors in group I recognized HLA-matched BLCL, C1R-A1 and C1R-A2 cells to a similar extent (average percentage of 65%). C1R-A1 and C1R-A2 cells were recognized and killed more efficiently than C1R-B8 and C1R-B35 cells ($P < 0.05$).
Influenza A virus-specific CD8+ T cells obtained from donors in group II lysed influenza virus-infected C1R-B27 cells efficiently. The percentage lysis that was observed was higher than HLA-matched BLCL cells or any of the other C1R cells expressing the other three alleles, including HLA-A*0201 which ranked second. Infected C1R-B8 cells were recognized relatively poorly.
CD8+ effector cells obtained from donors in group III did not exhibit a clear preferred HLA usage in the recognition of their target cells, although C1R-A1 cells were lysed more efficiently than C1R-A3, C1R-B8 or C1R-B35 cells.

HLA-A- and -B-restricted IFN-γ responses specific for influenza B virus
The IFN-γ response restricted by HLA-A and -B alleles was determined in PBMC of donors of group II (HLA-A1, HLA-A2, HLA-B8 and HLA-B27) stimulated with influenza B virus, in order to investigate whether HLA-B*2705 was also the preferred allele in response to another type of influenza virus. On average 41% IFN-γ+ CD8+ T cells were detected after restimulation with influenza B virus-infected HLA-matched BLCL cells. The majority of these cells recognized their epitopes in the context of HLA-B*0801 (22.7%), followed by the HLA-B*2705- (7.4%), HLA-A*0101- (2.4%) and HLA-A*0201-restricted (2.3%) responses. The proportion HLA-B*0801-restricted IFN-γ+ CD8+ T cells was significantly greater than the proportion C1R-A1- ($P = 0.05$) or C1R-A2- ($P = 0.05$) restricted IFN-γ+ CD8+ T cells. The HLA-A*0101- and HLA-A*0201-restricted IFN-γ responses were also significantly smaller than the HLA-B*2705-restricted response ($P = 0.017$ and $P = 0.002$ respectively).

IFN-γ and TNF-α expression in influenza A virus-specific CTL restricted by individual HLA-A and –B alleles
The expression of TNF-α was determined as a third functional parameter after restimulation of influenza A virus-stimulated PBMC with HLA-matched BLCL and HLA-transfected C1R cells. The ratio between % IFN-γ+ and % TNF-α+ cells within the CD8+ T cell fraction for each individual for the different HLA-transfected C1R cells and HLA-matched BLCL cells was calculated (figure 6). The ratio IFN-γ+ and TNF-α+ cells following stimulation with HLA-matched BLCL cells is on average 1.1, indicating that most virus-specific cells produce both cytokines upon restimulation. However, stimulation with HLA-transfected C1R cells resulted in higher ratios starting from 1.2 for C1R-B27 to 1.8 for C1R-A3, C1R-B8 and C1R-B35. The ratio observed for C1R-B8 was significantly different from the ratio observed with HLA-matched BLCL ($P = 0.024$) and indicate that some HLA-B8-restricted CTL (but also HLA-A3 and HLA-B35) produce IFN-γ and not TNF-α. Stimulation with C1R-A1 cells resulted in an average ratio IFN-γ+/TNF-α+ cells of 0.7, indicating that some HLA-A*0101-restricted influenza A virus-specific CTL

produce TNF-α but not IFN-γ. This ratio was significantly different from the average ratio of C1R-A2 ($P = 0.011$), C1R-A3 ($P = 0.072$), C1R-B8 ($P < 0.001$) and C1R-B35 ($P = 0.001$).

Discussion

The contribution of individual HLA-A and –B alleles in influenza virus-specific CTL responses was determined in groups of HLA class I matched donors. It was shown that influenza virus-stimulated PBMC of HLA-A- and –B-matched donors preferentially recognized certain HLA alleles, which depended on the type of virus studied. Furthermore, it was shown that the magnitude of CTL responses restricted by individual alleles can be influenced by the presence of other alleles, confirming data we have obtained recently with synthetic peptides (7). In addition, it was demonstrated that cytokine production profiles of CD8+ CTL depended on their HLA class I restriction elements.

Prior to investigation of CTL responses restricted by individual HLA-A and –B alleles the use of HLA-transfected C1R cells as APC was validated. The level of expression of HLA molecules was similar in the different C1R cells as measured with saturating amounts of antibody. These cells were also capable of processing and presenting influenza A virus-specific CTL epitopes to a similar extent as EBV-

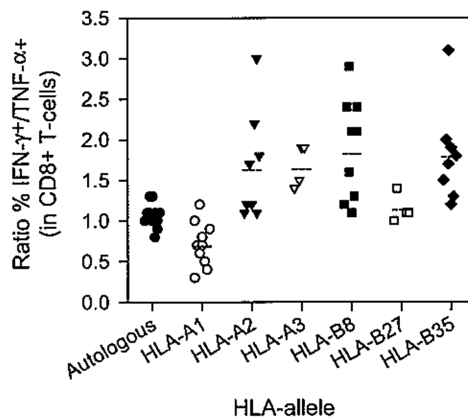

Figure 6: IFN-γ and TNF-α production in influenza virus-specific CD8+ T cells. After simultaneous detection of IFN-γ and TNF-α, the ratio was calculated of IFN-γ and TNF-α producing cells after restimulation of CD8+ T cells obtained after virus-specific expansion of PBMC with HLA-matched BLCL or C1R cells expressing individual HLA-A or –B alleles. Each symbol represents a study subject. The average ratio IFN-γ/TNF-α of all donors in group I-III is depicted (---).

transformed BLCL. In addition, only small differences in susceptibility of the individual C1R cells for infection with influenza A virus was found (between 55-80% infected), with the exception of C1R-B35. This however did not interfere with our ICS assay, since an excess number of stimulator cells was used to stimulate influenza virus-specific CTL in the *in vitro* stimulated PBMC cultures. In contrast to the use of BLCL matched for a single HLA allele, the use of C1R expressing single HLA alleles prevents possible competition for processing/presentation between overlapping epitopes (30). In EBV-transformed BLCL cells or C1R cells competition for available HLA-A or –B alleles between epitopes from influenza virus and EBV may occur. For example, an immunodominant HLA-B*0801-restricted CTL epitope of EBV ($EBNA3A_{325-333}$) could interfere with the presentation of HLA-B*0801-restricted influenza virus epitopes. However, influenza A virus-infected C1R-B8 cells were readily killed by HLA-B*0801-restricted influenza A virus-specific CTL indicating that competition between epitopes of these two viruses does not constitute a significant problem.
Using the HLA-transfected C1R cell lines it was shown that the influenza A virus-specific CTL response in these donors measured by intracellular IFN-γ staining was dominated by HLA-B*2705- and HLA-B*3501-restricted CTL in groups II and I/III respectively. Based on previous findings with synthetic peptides (7, 9) it was anticipated that the HLA-A*0201-restricted response, presenting the immunodominant epitope $M1_{58-66}$, would be recognized preferentially. Therefore, the observed hierarchy of virus-specific responses indicate that other yet unidentified HLA-B*2705- and HLA-B*3501-restricted epitopes exist. Recently an immunodominant HLA-B*3501-restricted epitope ($NP_{418-426}$) was identified (7, 9), which may have contributed to the preferred usage of HLA-B*3501 in the CTL response. In addition to the dominance of HLA-B27 and HLA-B35, a large proportion of T cells were specific for epitopes presented by HLA-A*0201 and HLA-A*0301. Most likely the presentation of the immunodominant epitope $M1_{58-66}$ has contributed to the dominance of the HLA-A*0201-restricted response. The large proportion of HLA-A*0301-restricted CTL indicate that more unknown immunodominant epitopes exist, since the HLA-A*0301-restricted $NP_{265-273}$ epitope has been shown to be subdominant in response to influenza A viruses (7). Indeed, a novel HLA-A3-restricted CTL epitope was identified recently, which could contribute to the influenza A virus-specific CTL response (29). Finally, the IFN-γ response specific for epitopes presented in the context of HLA-A*0101 and HLA-B*0801 contributed little to the overall influenza virus-specific CTL response, which is in agreement with previous work (30). The small contribution of these alleles to the overall CTL response could be caused by the presence of alleles HLA-B*2705 or HLA-B*3501 presenting immunodominant epitopes. This hypothesis was supported by a high proportion IFN-γ+ HLA-B*0801-restricted cells in an HLA-A*0101, HLA-B*0801 homozygous donor (data not shown). In addition, the HLA-B*0801-restricted response was lower in donors of group II (HLA-B27+ donors) than in donors in the two other groups (HLA-B35+ donors), which is in agreement with the poor recognition of the HLA-B*0801-restricted $NP_{380-388}$ epitope in HLA-B27+ individuals (7, 30). These data also indicate that more

immunodominant high affinity HLA-B*0801-restricted epitopes are not existing for influenza A virus.
Changes in epitope specificity of the virus-specific CTL response as a result of differences in HLA expression profiles were previously reported in mice infected with influenza A virus (3, 12, 28). In these studies, the expression of an H-2K^b allele (either transgenic or through breeding with C57BL mice), resulted in a reduced H-2^k-restricted response.
In a previous study (7), we reported a lower influenza A virus-specific immune response in HLA-A*0201 negative donors (group III). Also in the current study, the average number IFN-γ+ cells following restimulation with HLA-matched BLCL is lower in HLA-A*0201 negative donors than in HLA-A*0201 positive donors, although this difference was not statistically significant (P = 0.28). This is partly explained by the exclusion of two HLA-A*0201 negative donors with an HLA-B*3503 instead of an HLA-B*3501 genotype.
Using influenza B virus for the stimulation of PBMC we demonstrated that the preferential HLA usage is dependent on the virus studied. In contrast to the influenza A virus-specific CTL response, the HLA-B*0801-restricted response specific for influenza B virus, was highly immunodominant, followed by HLA-B*2705. The HLA-A*0101- and HLA-A*0201-restricted responses were shown to contribute little to the overall influenza B virus-specific response. It is difficult to correlate these responses to known (immunodominant) epitopes since only four influenza B virus CTL epitopes are known (25, 26), of which three are presented in the context of HLA-B8 (26). These data indicate that the available epitope repertoire determines the outcome of the CTL response and preferred HLA usage.
In contrast to other studies addressing the preferential use of HLA molecules in CTL responses we used virus-infected human cells expressing a single HLA allele which accounts for the full repertoire of CTL epitopes presented by these HLA molecules. The preferential usage of certain alleles in the virus-specific CTL response has also been reported for EBV-specific CTL responses. These data demonstrated that certain HLA class I alleles were dominantly recognized, such as HLA-B8, HLA-A11 or HLA-B44, while HLA-A1 was not (17, 22). For HIV-specific CTL responses it was shown with synthetic peptides that the HLA-A2-restricted response contributed hardly to the overall HIV-specific response (6, 11).
Most of these studies used IFN-γ production to identify epitope-specific CTL. However, our data suggest that CTL differ in their ability to produce cytokines depending on the epitopes recognized and/or the HLA molecules presenting these epitopes. Influenza A virus-specific HLA-A*0101-restricted CTL produced less IFN-γ and more TNF-α than CTL restricted by other HLA molecules. Therefore, some caution should be exercised in interpreting frequencies of CTL based on IFN-γ production alone. The difference in ratio IFN-γ/TNF-α production for HLA-A*0101-restricted CTL in comparison with other allele-restricted CTL is striking. These differences were not the result of the restimulation protocol with HLA-transfected C1R cell lines, since similar results were obtained after stimulation with an influenza virus-infected HLA-A*0101-homozygous BLCL, which also induced more TNF-α+ cells than IFN-γ+ CD8+ T cells (data not shown). To our knowledge this is the first study to identify differences in cytokine production in CD8+ CTL

effector cell populations. Previous studies identified functional differences between EBV-, CMV- and HIV-specific CTL, based on perforin and surface marker staining (1, 2). These studies also showed reduced killing of two HIV tetramer positive cell populations in comparison with a CMV tetramer positive population. Our data provides evidence that also acute viral infections, like influenza virus, induce functionally different CD8+ CTL populations. At present it is unclear what the underlying mechanism is for differential cytokine expression in virus-specific CTL and how epitope specificity and the HLA molecules control this. Additional studies are required to further characterize these functional differences in CTL function and to investigate the implications of differential cytokine expression.
Thus collectively the present study has shown in donors of well-defined HLA genotypes that; 1) In response to virus infection CTL responses are induced which use certain HLA molecules preferentially, depending on the available repertoire of CTL epitopes, 2) The magnitude of CTL responses restricted by a single HLA-allele can be influenced by the presence of another HLA-A or –B allele, and 3) CTL exhibit differential cytokine expression depending on their epitope specificity and/or HLA-restriction.

References

1. **Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael, and S. L. Rowland-Jones.** 2002. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat. Med. **8:**379-85.
2. **Appay, V., D. F. Nixon, S. MDDonahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael, and S. L. Rowland-Jones.** 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J. Exp. Med. **192:**63-75.
3. **Belz, G. T., P. G. Stevenson, and P. C. Doherty.** 2000. Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. J. Immunol. **165:**2404-9.
4. **Bender, B. S., T. Croghan, L. Zhang, and P. A. Small.** 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. **175:**1143-1145.
5. **Berzofsky, J. A.** 1988. Immunodominance in T lymphocyte recognition. Immunol. Lett. **18:**83-92.
6. **Betts, M. R., J. P. Casazza, B. A. Patterson, S. Waldrop, W. Trigona, T. M. Fu, F. Kern, L. J. Picker, and R. A. Koup.** 2000. Putative immunodominant human immunodeficiency virus-specific CD8(+) T- cell responses cannot be predicted by major histocompatibility complex class I haplotype. J. Virol. **74:**9144-51.

7. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. The magnitude and specificity of influenza A virus-specific cytotoxic T lymphocyte responses in humans is related to HLA-A and -B phenotype. J. Virol. **76:**582-90.
8. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. J. Virol. **76:**2567-72.
9. **Boon, A. C., G. de Mutsert, D. van Baarle, D. J. Smith, A. S. Lapedes, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2003. Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T cells. submitted for publication.
10. **Boon, A. C., E. Fringuelli, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. M. Iorio, G. F. Rimmelzwaan, and A. D. Osterhaus.** 2002. Influenza A virus specific T cell immunity in humans during aging. Virology. **299:**100-8.
11. **Day, C. L., A. K. Shea, M. A. Altfeld, D. P. Olson, S. P. Buchbinder, F. M. Hecht, E. S. Rosenberg, B. D. Walker, and S. A. Kalams.** 2001. Relative dominance of epitope-specific cytotoxic T lymphocyte responses in human immunodeficiency virus type 1-infected persons with shared HLA alleles. J. Virol. **75:**6279-91.
12. **Doherty, P. C., W. E. Biddison, J. R. Bennink, and B. B. Knowles.** 1978. Cytotoxic T cell responses in mice infected with influenza and vaccinia viruses vary in magnitude with H-2 genotype. J. Exp. Med. **148:**534-43.
13. **Elkington, R., S. Walker, T. Crough, M. Menzies, J. Tellam, M. Bharadwaj, and R. Khanna.** 2003. Ex vivo profiling of CD8+-T cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers. J. Virol. **77:**5226-40.
14. **Ennis, F. A., A. H. Rook, Y. H. Qi, G. C. Schild, D. Riley, R. Pratt, and C. W. Potter.** 1981. HLA restricted virus-specific cytotoxic T lymphocyte responses to live and inactivated influenza vaccines. Lancet. **2:**887-91.
15. **Gavioli, R., M. G. Kurilla, P. O. de Campos-Lima, L. E. Wallace, R. Dolcetti, R. J. Murray, A. B. Rickinson, and M. G. Masucci.** 1993. Multiple HLA A11-restricted cytotoxic T lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. J. Virol. **67:**1572-8.
16. **Geretti, A. M., C. A. Van Els, C. A. Van Baalen, M. C. Poelen, and A. D. Osterhaus.** 1993. Preservation of phenotype and function of positively selected virus- specific CD8+ T lymphocytes following anti-Fab detachment from immunomagnetic beads. J. Immunol. Methods. **161:**129-33.
17. **Houssaint, E., X. Saulquin, E. Scotet, and M. Bonneville.** 2001. Immunodominant CD8 T cell response to Epstein-Barr virus. Biomed Pharmacother. **55:**373-80.
18. **Masurel, N., P. Ophof, and P. de Jong.** 1981. Antibody response to immunization with influenza A/USSR/77.(H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. J. Hyg. (Lond). **87:**201-9.

19. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare.** 1983. Cytotoxic T cell immunity to influenza. N. Engl. J. Med. **309:**13-7.
20. **Novitsky, V., H. Cao, N. Rybak, P. Gilbert, M. F. McLane, S. Gaolekwe, T. Peter, I. Thior, T. Ndung'u, R. Marlink, T. H. Lee, and M. Essex.** 2002. Magnitude and frequency of cytotoxic T lymphocyte responses: identification of immunodominant regions of human immunodeficiency virus type 1 subtype C. J. Virol. **76:**10155-68.
21. **Palmer, D., W. Dowdle, M. Coleman, and G. Schild** 1975. Haemagglutination inhibition test, p. 25-62. *In* Anonymous (ed.), Advanced laboratory techniques for influenza diagnosis. Procedural Guide. US Dept. Hlth. ED., Atlanta.
22. **Rickinson, A. B., and D. J. Moss.** 1997. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. Annu. Rev. Immunol. **15:**405-31.
23. **Rimmelzwaan, G. F., M. Baars, E. C. Claas, and A. D. Osterhaus.** 1998. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J. Virol. Methods. **74:**57-66.
24. **Rimmelzwaan, G. F., N. Nieuwkoop, A. Brandenburg, G. Sutter, W. E. Beyer, D. Maher, J. Bates, and A. D. Osterhaus.** 2000. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine. **19:**1180-7.
25. **Robbins, P. A., L. A. Lettice, P. Rota, J. Santos-Aguado, J. Rothbard, A. J. McMichael, and J. L. Strominger.** 1989. Comparison between two peptide epitopes presented to cytotoxic T lymphocytes by HLA-A2. Evidence for discrete locations within HLA-A2. J. Immunol. **143:**4098-103.
26. **Robbins, P. A., P. A. Rota, and S. Z. Shapiro.** 1997. A broad cytotoxic T lymphocyte response to influenza type B virus presented by multiple HLA molecules. Int. Immunol. **9:**815-23.
27. **Sedlik, C., G. Dadaglio, M. F. Saron, E. Deriaud, M. Rojas, S. I. Casal, and C. Leclerc.** 2000. In vivo induction of a high-avidity, high-frequency cytotoxic T lymphocyte response is associated with antiviral protective immunity. J. Virol. **74:**5769-75.
28. **Tourdot, S., and K. G. Gould.** 2002. Competition between MHC class I alleles for cell surface expression alters CTL responses to influenza A virus. J. Immunol. **169:**5615-21.
29. **Trojan, A., M. Urosevic, J. Hummerjohann, R. Giger, U. Schanz, and R. A. Stahel.** 2003. Immune Reactivity Against a Novel HLA-A3-Restricted Influenza Virus Peptide Identified by Predictive Algorithms and Interferon-gamma Quantitative PCR. J Immunother. **26:**41-6.
30. **Tussey, L. G., S. Rowland-Jones, T. S. Zheng, M. J. Androlewicz, P. Cresswell, J. A. Frelinger, and A. J. McMichael.** 1995. Different MHC class I alleles compete for presentation of overlapping viral epitopes. Immunity. **3:**65-77.
31. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus

(H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J. Virol. **74:**6800-6807.
32. **Voeten, J. T., G. F. Rimmelzwaan, N. J. Nieuwkoop, R. A. Fouchier, and A. D. Osterhaus.** 2001. Antigen processing for MHC class I restricted presentation of exogenous influenza A virus nucleoprotein by B-lymphoblastoid cells. Clin. Exp. Immunol. **125:**423-31.
33. **Yewdell, J. W., and J. R. Bennink.** 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu. Rev. Immunol. **17:**51-88.
34. **Zemmour, J.** 1996. Inefficient assembly limits transport and cell surface expression of HLA-Cw4 molecules in C1R. Tissue Antigens. **48:**651-61.
35. **Zemmour, J., A. M. Little, D. J. Schendel, and P. Parham.** 1992. The HLA-A,B "negative" mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. J. Immunol. **148:**1941-8.

Chapter 4

Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes

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Journal of Virology 2002; 76(5):2567-2572

Abstract

Here, we describe a new HLA-B*3501-restricted cytotoxic T lymphocyte (CTL) epitope in the influenza A virus (H3N2) nucleoprotein, which was found to exhibit a high degree of variation at nonanchor residues. The influenza virus variants emerged in chronological order, and CTL directed against old variants failed to recognize more recent strains of influenza A virus, indicating an escape from CTL immunity.

CD8+ cytotoxic T lymphocytes (CTL) contribute to the control of viral infections by recognizing antigenic peptides of viral proteins presented by major histocompatibility complex (MHC) class I molecules on infected cells. The specific recognition of these MHC-peptide complexes by CTL may lead to the elimination of virus-infected cells. One of the mechanisms exploited by viruses to evade recognition by CTL (19, 23, 28), involves antigenic variation in CTL epitopes or mutations in sequences flanking these epitopes. Antigenic variation in CTL epitopes, resulting in evasion from immune surveillance by specific CTL has been described for several viruses causing chronic infections including Epstein-Barr virus (EBV) (5,

Table 1: Variation in HLA-B35-restricted influenza A virus epitope $NP_{418-426}$

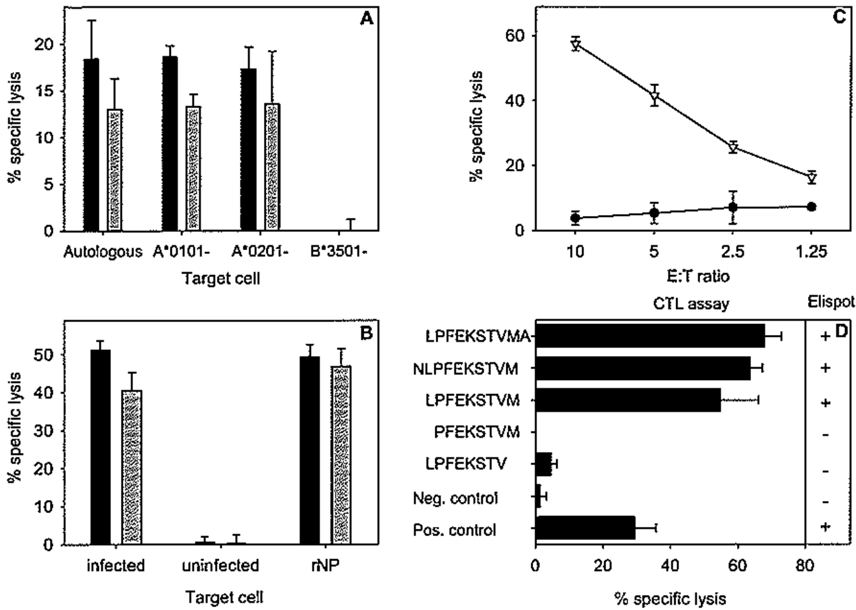
Sequence[a]	Yr(s) of isolation	Virus subtype	No. of viruses[b]	Epitope Name
LPFDRPTIM	Before 1933	H1N1	1	
-----T---	1934	H1N1	1	
----KT---	1940-1957	H1N1/H2N2	6	
----K----	1957-1972	H2N2/H3N2	7	$NP_{418-426}$/1957
----KS---	1972-1978	H3N2	12	$NP_{418-426}$/1972
----KS-V-	1977	H3N2	1	
---EKS---	1983	H3N2	1	
---EKS-V-	1980 – present	H3N2	35	$NP_{418-426}$/1980

[a] A dash indicates identity with amino acid position with the first sequence.
[b] Number of viruses with the reported sequence in the influenza sequence database (http://www.flu.lanl.gov). Influenza A (H1N1) viruses were excluded from the analysis from 1977, the year of reintroduction of viruses with this subtype.

8, 9, 12), human immunodeficiency virus (4, 7, 13, 14, 22, 26), hepatitis B virus (1, 2) and hepatitis C virus (6, 35).

Recently, mutations were also found at the anchor residue of an HLA-B*2705-restricted epitope of the influenza A virus nucleoprotein (NP), which consisted of amino acid 383 to 391 ($NP_{383-391}$). Both the $R_{384}G$ and the $R_{384}K$ mutation abolished class I-restricted presentation and allowed for escape from CTL recognition (33). Thus, these viruses, which cause acute infections in a significant portion of the human population annually, can escape from immune surveillance by CTL in addition to escape from neutralizing antibodies (antigenic drift and antigenic shift).

For the identification of new CTL epitopes, CD3+ and CD8+ T cell clones were generated by limiting dilution (33), from peripheral blood mononuclear cells (PBMC) of an HLA-A*0101-, -A*0201-, -B*0801-, B*3501-positive donor after *in vitro* stimulation with influenza virus Resvir-9, a reassortant vaccine strain of A/Nanchang/933/95 (3). For the initial specificity testing of the T cell clones, an enzyme-linked immunospot assay was used, as described previously (3), with infected autologous B-lymphoblastoid cell line (BLCL) as stimulator cells.



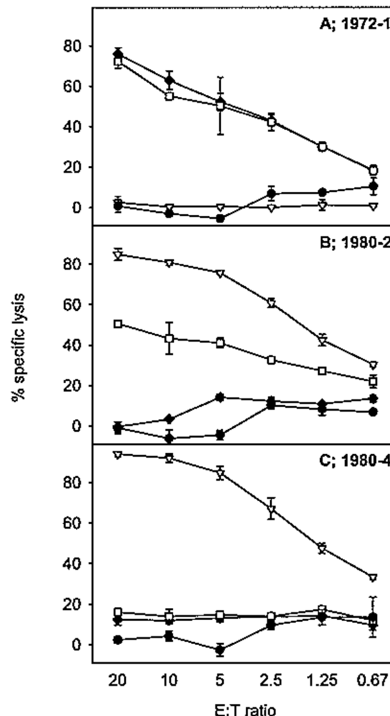


Figure 2: $NP_{418-426}$ variant epitope specificity of CTL clones.
Target cells (BLCL) were incubated overnight with 5 µM concentrations of the HLA-B*3501-restricted $NP_{418-426}$ epitope variants, namely, $NP_{418-426}$/1980 (▽), $NP_{418-426}$/1972 (□) and $NP_{418-426}$/1957 (◆), or were untreated (●) and used as a negative control. Clones 1972-1 (A), 1980-2 (B) and 1980-4 (C), obtained from different donors, were added at different effector-to-target cell ratios and specific lysis was calculated. The results, given as the percent specific lysis (mean ± SD), are representative of multiple assays.

Figure 1: Characterization of an HLA-B*3501-restricted epitope and CTL clone (1980-1).
(A) HLA class I restriction of CTL clone 1980-1 was determined by partially mismatched target cells (BLCL) infected with influenza A (H3N2) virus (Resvir-9) in a ^{51}Cr release assay at effector-to-target cell (E:T) ratios of 10:1 (black bars) and 5:1 (grey bars) as described previously (3). Autologous BLCL cells (HLA-A*0101, -A*0201, -B*0801,and -B*3501) and BLCL cells mismatched for a single HLA class I molecule (A*0101-, A*0201- and B*3501-) were used. All BLCL expressed HLA-B*0801. The percent lysis of uninfected target cells was subtracted from the percent lysis of target cells infected with influenza virus. (B) Protein specificity of an HLA-B*3501-restricted CTL clone. HLA-B*3501+ target cells were infected with influenza A (H3N2) virus (Resvir-9) or were left uninfected and used as a positive and negative control respectively. In addition, HLA-B*3501+ target cells were incubated overnight with 100 μg of bacterial expressed rNP of A/Netherlands/18/94 (A/H3N2)/ml, as previously described (33). The target cells were used in a ^{51}Cr-release assay with CTL clone 1980-1 at E:T ratios of 10:1 (black bars) and 5:1 (grey bars). (C) A 9-mer peptide ($NP_{418-426}$, LPFEKSTVM) (▽), predicted with a HLA binding prediction program (25) was loaded onto HLA-B*3501+ BLCL cells and used as target cells in a ^{51}Cr-release assay with the T cell clone 1980-1 at the indicated E:T ratios. Untreated BLCL cells (●) were included as a negative control. (D) Minimal epitope mapping of HLA-B*3501-restricted epitope. Peptides were synthesized based on the initial 9-amino-acid sequence LPFEKSTVM that were extended or truncated at the C- or N-terminal end of the $NP_{418-426}$ epitope in order to determine the minimal epitope. BLCL cells loaded with 5 μM concentrations of the different peptides were used as target cells in a CTL assay at an E:T ratio of 5:1, or applied to stimulate the CTL clone (1980-1) in an enzyme-linked immunospot assay . A (plus) indicates gamma-interferon (IFN-γ) production by the CTL clone, while (minus) means no production of IFN-γ. Influenza virus (Resvir-9)-infected BLCL cells and uninfected BLCL cells were included in both assays as positive and negative controls, respectively. Percent lysis is given ± SD and the results are representative of multiple experiments.

Clone 1980-1 was found to recognize a yet unidentified epitope in an HLA-B*3501- and HLA-B*3503-restricted fashion, as demonstrated with HLA matching and nonmatching infected BLCL as target cells (figure 1a, also data not shown). This clone was specific for the NP of influenza A virus (H3N2) since it lysed target cells incubated with recombinant NP (rNP) (figure 1b) as described previously (3, 34). Using a CTL epitope prediction program (25) (http://www.umds.ac.uk/tissue), putative HLA-B*3501-restricted epitopes in the NP of influenza A virus (H3N2) were predicted. The 9-mer with the highest ranking, LPFEKSTVM ($NP_{418-426}$/1980), was synthesized and was found to be recognized by clone 1980-1 in a CTL assay (figure 1c). Removal of the C-terminal methionine abolished recognition by the T cell clone 1980-1 (figure 1d). A similar result was observed when the lysine at the N terminus from the 9-mer epitope LPFEKSTVM was removed. Addition of one amino acid at the N or C terminus of the epitope did not significantly improve recognition of target cells, indicating that $NP_{418-426}$ was the minimal epitope.

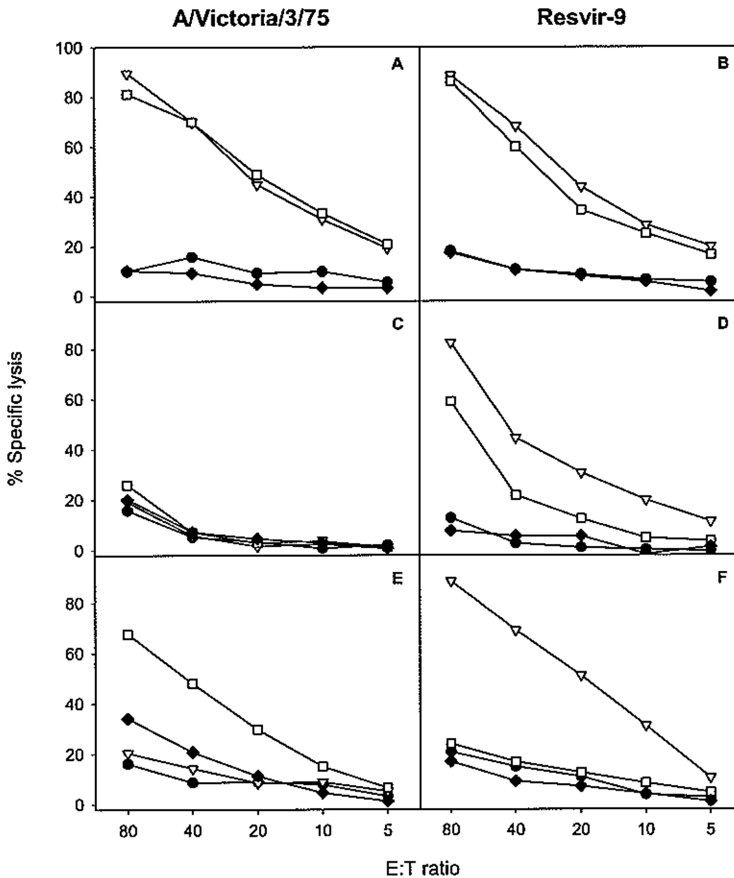


Figure 3: Epitope specificity of the polyclonal response following *in vitro* stimulation of PBMC cryopreserved in the year 2001 with influenza A viruses (H3N2) containing different variants of the epitope.
PBMC of HLA-B35+ donors, aged between 30 and 50 years, were stimulated *in vitro* with A/Victoria/3/75 (A, C, and E), containing the $NP_{418-426}$/1972 variant, or Resvir-9 (B, D, and F) containing the $NP_{418-426}$/1980 epitope as described previously (3). After 8 days, the effector cells were tested for lytic activity against target cells incubated with the peptides corresponding to $NP_{418-426}$/1980 (▽), $NP_{418-426}$/1972 (□) and $NP_{418-426}$/1957 (◆). Untreated target cells (●) were included in each assay as a negative control. Mean percentages of specific lysis of two independently repeated experiments are shown for donors 5972 (A and B), 5017 (C and D), and 5991 (E and F).

Experiments with rNPs from different virus strains indicated that clone 1980-1 did not react with the rNP obtained from A/Hong Kong/2/68 (H3N2) (data not shown). Therefore known $NP_{418-426}$ sequences of influenza A viruses obtained from the influenza sequence database (http://www.flu.lanl.gov) were compared. It was found that the amino acid sequence in the epitope varied at four positions and that these variants emerged in chronological order. All variants and their designations are shown in Table 1. Although the strains used for NP sequence comparison were not controlled for passage history of the strains (egg grown or passaged in mammalian cell lines), it is unlikely that this has biased the analysis, since no immediate selective pressure was observed on the NP by adaptation in eggs, as was for the hemagglutinin (29).
A T2 cell line expressing HLA-B*3501 (30) was used to assess the affinity of the $NP_{418-426}$ epitopes for the HLA-B*3501 molecule as previously described (32). The concentration of peptide necessary to inhibit the signal (mean fluorescence intensity [MFI]) of the FITC-labeled reference peptide $LPSC_{FL}ADVEF$ (20) by 50% (IC_{50}) was determined. The mean IC_{50} of the $NP_{418-426}$/1980 epitope was 1.1 μM, while the mean IC_{50} values of the $NP_{418-426}$/1972 and $NP_{418-426}$/1957 were 1.5 and 1.6 μM

Table 2: Binding affinity of $NP_{418-426}$ peptide to HLA-B*3501

Peptide	Sequence	IC_{50} (μM)[a]
$NP_{418-426}$/1957	LPFDKPTIM	1.3 – 2.0
$NP_{418-426}$/1972	LPFDKSTIM	1.1 – 2.0
$NP_{418-426}$/1980	LPFEKSTVM	0.9 - 1.3
$M1_{128-135}$[b]	ASCMGLIY	> 20

[a] Binding capacity to HLA-B*3501 is expressed as the concentration (μM) of peptide able to inhibit binding of the FL-labeled reference peptide with 50% (32) of two assays.
[b] HLA-B35-restricted influenza virus epitope from the matrix protein with a reported low binding affinity (10).

respectively. This indicates that all three epitopes bound strongly and in the same order of magnitude to HLA-B*3501 (Tab. 2), which suggests that the three peptides represent CTL epitopes. For comparison, the IC_{50} value of a previously described HLA-B35-restricted influenza A virus epitope $M1_{128-135}$ was determined and found to be higher than the highest concentration used (20 μM).
The frequency of peptide-specific CTL precursors was determined for the $NP_{418-426}$/1980, $NP_{418-426}$/1972, and $NP_{418-426}$/1957 variant of the epitope and found to differ considerably among four HLA-B35+ donors (Tab. 3). The average frequency of $NP_{418-426}$/1980-specific cells was 1 in 7426, ranging from 1 in 4073 to 1 in 15485, which is relatively high compared to the frequencies of other CTL epitopes of influenza A virus (3), indicating that $NP_{418-426}$/1980 is an immunodominant epitope. The mean frequency of $NP_{418-426}$/1972-specific cells is much lower (1 in 16226),

however one donor exhibited a high number of $NP_{418\text{-}426}$/1972-specific cells. For the $NP_{418\text{-}426}$/1957 epitope, low to undetectable numbers of specific cells were detected. Based on the prevalence of the sequences (Tab. 1), we decided to focus on the $NP_{418\text{-}426}$/1980, $NP_{418\text{-}426}$/1972, $NP_{418\text{-}426}$/1957 epitopes. Since mutations in the $NP_{418\text{-}426}$ epitope emerged in an evolutionary fashion, we speculated that CTL immunity directed against these epitopes was at the basis of the selection and/or emergence of mutant viruses. To test this hypothesis, more T cell clones were raised against historic (A/Victoria/3/75) and recent (Resvir-9) viruses by using PBMC from HLA-B35+ donors obtained in the year 2001. CTL clone 1972-1 directed against the

Table 3: $NP_{418\text{-}426}$ peptide-specific CTL frequencies in PBMC

Donor	$NP_{418\text{-}426}$/1980	$NP_{418\text{-}426}$/1972	$NP_{418\text{-}426}$/1957
5017	1/1885	1/17921	-[a]
5972	1/4073	1/5054	1/86550
2384	1/15485	1/17329	1/10108
5991	1/7542	1/24600	1/24600
Mean	1/7246	1/16226	1/40419

[a]No specific spots detected

$NP_{418\text{-}426}$/1972 epitope failed to recognize the $NP_{418\text{-}426}$/1980 epitope (figure 2a). Similarly, three out of five CTL clones (1980-1, 1980-4, and 1980-5) directed against the $NP_{418\text{-}426}$/1980 epitope failed to react with the $NP_{418\text{-}426}$/1957 and $NP_{418\text{-}426}$/1972 epitopes (figure 2c, also data not shown). With the other two CTL clones (1980-2, and 1980-3) some cross-reactivity was observed with the epitopes in older influenza A virus strains (figure 2b, also data not shown). The absence of recognition of the $NP_{418\text{-}426}$/1980 epitope by CTL directed against older variants was also demonstrated with PBMC obtained from two donors stimulated *in vitro* with influenza virus A/Victoria/3/75 (figure 3e, also data not shown). These PBMC did however recognize the homologous $NP_{418\text{-}426}$/1972 epitope. PBMC of some donors displayed cross-reactivity with both variants after stimulation with A/Victoria/3/75 or Resvir-9 (figure 3). This cross-reactivity can be explained by the expansion of cross-reactive CTL in PBMC, a phenomenon previously described in C57BL/10 mice infected with different strains of influenza A virus (15). Finally, one donor (donor 5017) did not respond at all after stimulation with A/Victoria/3/75 (figure 3c).

To exclude the influence of consecutive natural infections with various influenza A viruses on the $NP_{418\text{-}426}$-specific CTL response, we obtained HLA-A2+, HLA-B35+ PBMC cryopreserved between the years 1982 and 1984. Since this occurred shortly after the introduction of the $NP_{418\text{-}426}$/1980 variant epitope, the chance of an infection with an $NP_{418\text{-}426}$/1980 variant virus in these donors is relatively small. The $NP_{418\text{-}426}$/1980 epitope was not recognized by the PBMC of these donors (donor MHO and JHO), (figure 4), after stimulation with both a recent strain of influenza virus (Resvir-9) and A/Victoria/3/75, which contained the $NP_{418\text{-}426}$/1972 epitope. The

lack of $NP_{418\text{-}426}$/1980-specific activity was not caused by the absence of virus-specific CTL activity, since the $NP_{418\text{-}426}$/1972 variant epitope and the conserved HLA-A*0201-restricted $M1_{58\text{-}66}$ epitope were recognized.

Based on these findings, we argue that in addition to the variation in the HLA-B8- and HLA-B*2705-restricted epitopes, $NP_{380\text{-}388}$ and $NP_{383\text{-}391}$ respectively, the variation in this newly identified HLA-B*3501-restricted $NP_{418\text{-}426}$ epitope is driven by CTL immunity leading to escape from recognition by these CTL. The variation in

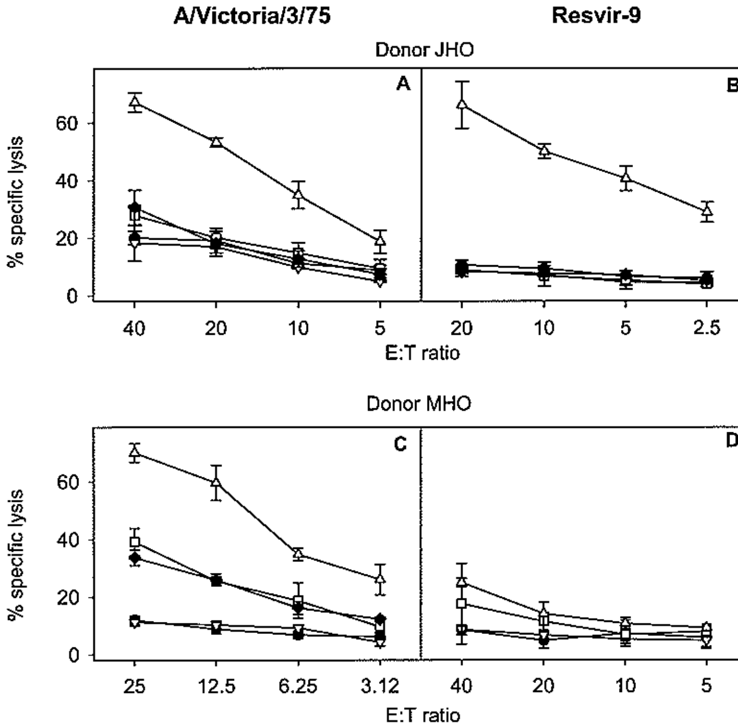


Figure 4: CTL responsiveness after stimulation of PBMC cryopreserved between 1982 and 1984 with influenza A viruses (H3N2) containing the $NP_{418\text{-}426}$/1980 or $NP_{418\text{-}426}$/1972 variant epitope.

PBMC from two HLA-A2+, -B35+ donors (donors JHO and MHO) were stimulated *in vitro* with A/Victoria/3/75 (A and C) containing the $NP_{418\text{-}426}$/1972 variant epitope, or Resvir-9 (B and D), containing the $NP_{418\text{-}426}$/1980 variant epitope. The lytic activities of the effector cells were determined after 8 days of culture against target cells incubated with 5 μM concentrations of peptides corresponding to $NP_{418\text{-}426}$/1980 (▽), $NP_{418\text{-}426}$/1972 (□), $NP_{418\text{-}426}$/1957 (◆) or $M1_{58\text{-}66}$ epitope (△), and a negative control (●) at different effector-to-target cell ratios. $NP_{418\text{-}426}$/1957 was not determined for PBMC of donor MHO after stimulation with Resvir-9. The mean percentage specific lysis ± SD was given for one experiment.

the $NP_{380-388}$ and $NP_{383-391}$ epitopes were found at the anchor residues ($R_{384}G$ mutation) of the respective 9-mers (34). In the $NP_{418-426}$ epitope, the anchor residues for binding to HLA-B35 were perfectly conserved in all virus strains isolated and sequenced since 1933. It can be speculated that variation at these residues is restricted by functional constraints. For example, an $R_{267}A$ mutation within an HLA-A3-restricted epitope has been shown to affect RNA binding by NP (11). Since the NP of A/Hong Kong/2/68 containing the $NP_{418-426}$/1957 epitope, and the NP of A/Netherlands/18/94 containing the $NP_{418-426}$/1980 epitope function equally well (34) and both viruses grow to comparable titers, it is unlikely that the mutations in the $NP_{418-426}$ were selected based on improved fitness of these viruses.

HLA-B35-positive individuals constitute a significant portion of the human population, ranging from 5% in Orientals to 10% in Caucasians (21). The immune pressure mediated by CTL in these individuals recognizing the $NP_{418-426}$ epitope may have contributed to the emergence of escape mutant viruses from the quasi species of influenza viruses and their continued circulation. As a result of the immunodominant nature of the epitope, the CTL response might have been oligoclonal in HLA-B35+ individuals, allowing for the selection of mutant viruses. Alternatively, other unknown epitopes may overlap with $NP_{418-426}$, further contributing to the selection pressure. The variants are maintained in HLA-B35-negative individuals because the mutations in the $NP_{418-426}$ epitope did not reduce the fitness of these viruses. It is of interest that the infection of mice transgenic for a single T cell receptor (TCR) specific for the H-2D^b-restricted $NP_{366-374}$ epitope of influenza A virus resulted in the emergence of viruses containing amino acid mutations in this epitope, which impaired the presentation of viral peptides by MHC class I molecules or interfered with TCR recognition (27). Other mutations at nonanchor residues have been described in sporadic virus isolates, and their effect on T cell recognition was not studied (24). A mutation in a CTL epitope ($NS1_{122-130}$) in the (not naturally occurring) high yield vaccine strain of influenza virus A/Texas/36/91 (31), inhibited recognition by a CTL clone. Other mechanisms based on mutations in CTL epitopes contributing to reduction or elimination of CTL recognition have been described, like "original antigenic sin" (18), antagonism (16, 17), and anergy. Although 'original antigenic sin' and anergy were not investigated in great detail, in one of the PBMC samples, obtained in 1982, a response was found that was specific for the $NP_{418-426}$/1972 epitope but not for the homologous $NP_{418-426}$/1980 epitope used for stimulation.

In conclusion, a new variable CTL epitope was identified in the NPs of influenza A viruses. Epidemiological and immunological evidence indicates that the variation found in this epitope was the result of antigenic drift resulting from immune pressure mediated by specific CTL. Thus, in addition to the introduction of mutations in the surface glycoproteins, which allows for the escape from antibody-mediated immunity, further evidence which shows that influenza viruses can escape from CTL-mediated immunity is accumulating. This would make these viruses masters in disguise, partially explaining their relative success in infecting a large portion of the human population every year for the past several decades.

References

1. **Bertoletti, A., A. Costanzo, F. V. Chisari, M. Levrero, M. Artini, A. Sette, A. Penna, T. Giuberti, F. Fiaccadori, and C. Ferrari.** 1994. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. J. Exp. Med. **180:**933-943.
2. **Bertoletti, A., A. Sette, F. V. Chisari, A. Penna, M. Levrero, M. De Carli, F. Fiaccadori, and C. Ferrari.** 1994. Natural variants of cytotoxic epitopes are T cell receptor antagonists for antiviral cytotoxic T cells. Nature. **369:**407-410.
3. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. The magnitude and specificity of influenza A virus-specific CTL responses in humans is related to HLA-A and -B phenotype. J. Virol. **76**:582-590.
4. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. **3:**205-211.
5. **Burrows, J. M., S. R. Burrows, L. M. Poulsen, T. B. Sculley, D. J. Moss, and R. Khanna.** 1996. Unusually high frequency of Epstein-Barr virus genetic variants in Papua New Guinea that can escape cytotoxic T cell recognition: implications for virus evolution. J. Virol. **70:**2490-2496.
6. **Chang, K. M., B. Rehermann, J. G. McHutchison, C. Pasquinelli, S. Southwood, A. Sette, and F. V. Chisari.** 1997. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. J. Clin. Invest. **100:**2376-2385.
7. **Couillin, I., B. Culmann-Penciolelli, E. Gomard, J. Choppin, J. P. Levy, J. G. Guillet, and S. Saragosti.** 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein. J. Exp. Med. **180:**1129-1134.
8. **de Campos-Lima, P. O., R. Gavioli, Q. J. Zhang, L. E. Wallace, R. Dolcetti, M. Rowe, A. B. Rickinson, and M. G. Masucci.** 1993. HLA-A11 epitope loss isolates of Epstein-Barr virus from a highly A11+ population. Science. **260:**98-100.
9. **de Campos-Lima, P. O., V. Levitsky, J. Brooks, S. P. Lee, L. F. Hu, A. B. Rickinson, and M. G. Masucci.** 1994. T cell responses and virus evolution: loss of HLA A11-restricted CTL epitopes in Epstein-Barr virus isolates from highly A11-positive populations by selective mutation of anchor residues. J. Exp. Med. **179:**1297-1305.
10. **Dong, T., D. Boyd, W. Rosenberg, N. Alp, M. Takiguchi, A. McMichael, and S. Rowland-Jones.** 1996. An HLA-B35-restricted epitope modified at an anchor residue results in an antagonist peptide. Eur. J. Immunol. **26:**335-339.

11. **Elton, D., L. Medcalf, K. Bishop, D. Harrison, and P. Digard.** 1999. Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding. J. Virol. **73:**7357-7367.
12. **Gottschalk, S., C. Y. Ng, M. Perez, C. A. Smith, C. Sample, M. K. Brenner, H. E. Heslop, and C. M. Rooney.** 2001. An Epstein-Barr virus deletion mutant associated with fatal lymphoproliferative disease unresponsive to therapy with virus-specific CTLs. Blood. **97:**835-843.
13. **Goulder, P. J., C. Brander, Y. Tang, C. Tremblay, R. A. Colbert, M. M. Addo, E. S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S. I. Pelton, S. K. Burchett, K. McIntosh, B. T. Korber, and B. D. Walker.** 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. Nature. **412:**334-338.
14. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T lymphocyte response associated with progression to AIDS. Nat. Med. **3:**212-217.
15. **Haanen, J. B., M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher.** 1999. Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. J. Exp. Med. **190:**1319-1328.
16. **Klenerman, P., U. C. Meier, R. E. Phillips, and A. J. McMichael.** 1995. The effects of natural altered peptide ligands on the whole blood cytotoxic T lymphocyte response to human immunodeficiency virus. Eur. J. Immunol. **25:**1927-1931.
17. **Klenerman, P., S. Rowland-Jones, S. McAdam, J. Edwards, S. Daenke, D. Lalloo, B. Koppe, W. Rosenberg, D. Boyd, A. Edwards, and et al.** 1994. Cytotoxic T cell activity antagonized by naturally occurring HIV-1 Gag variants. Nature. **369:**403-407.
18. **Klenerman, P., and R. M. Zinkernagel.** 1998. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. Nature. **394:**482-485.
19. **Koup, R. A.** 1994. Virus escape from CTL recognition. J. Exp. Med. **180:**779-782.
20. **Mandruzzato, S., V. Stroobant, N. Demotte, and P. van der Bruggen.** 2000. A human CTL recognizes a caspase-8-derived peptide on autologous HLA-B*3503 molecules and two unrelated peptides on allogeneic HLA-B*3501 molecules. J. Immunol. **164:**4130-4134.
21. **Marsh, S. G. E., P. Parham, and L. D. Barber** 2000. The HLA Factsbook, p. 182-186. *In* Anonymous (ed.), Factsbook Series. Academic Press, London, United Kingdom.
22. **McAdam, S., P. Klenerman, L. Tussey, S. Rowland-Jones, D. Lalloo, R. Phillips, A. Edwards, P. Giangrande, A. L. Brown, F. Gotch, and et al.** 1995. Immunogenic HIV variant peptides that bind to HLA-B8 can fail to stimulate cytotoxic T lymphocyte responses. J. Immunol. **155:**2729-2736.
23. **Oldstone, M. B.** 1997. How viruses escape from cytotoxic T lymphocytes: molecular parameters and players. Virology. **234:**179-185.

24. **Parker, C. E., and K. G. Gould.** 1996. Influenza A virus - A model for viral antigen presentation to cytotoxic T lymphocytes. Semin. Virol. **7**:61-73.
25. **Parker, K. C., M. A. Bednarek, and J. E. Coligan.** 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. **152**:163-175.
26. **Price, D. A., U. C. Meier, P. Klenerman, M. A. Purbhoo, R. E. Phillips, and A. K. Sewell.** 1998. The influence of antigenic variation on cytotoxic T lymphocyte responses in HIV-1 infection. J. Mol. Med. **76**:699-708.
27. **Price, G. E., R. Ou, H. Jiang, L. Huang, and D. Moskophidis.** 2000. Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia. J. Exp. Med. **191**:1853-1867.
28. **Sewell, A. K., D. A. Price, A. Oxenius, A. D. Kelleher, and R. E. Phillips.** 2000. Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. Stem Cells. **18**:230-244.
29. **Shu, L. L., W. J. Bean, and R. G. Webster.** 1993. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. J. Virol. **67**:2723-2729.
30. **Takamiya, Y., C. Schonbach, K. Nokihara, M. Yamaguchi, S. Ferrone, K. Kano, K. Egawa, and M. Takiguchi.** 1994. HLA-B*3501-peptide interactions: role of anchor residues of peptides in their binding to HLA-B*3501 molecules. Int. Immunol. **6**:255-261.
31. **Terajima, M., J. Jameson, J. E. Norman, J. Cruz, and F. A. Ennis.** 1999. High-yield reassortant influenza vaccine production virus has a mutation at an HLA-A 2.1-restricted CD8+ CTL epitope on the NS1 protein. Virology. **259**:135-140.
32. **van der Burg, S. H., E. Ras, J. W. Drijfhout, W. E. Benckhuijsen, A. J. Bremers, C. J. Melief, and W. M. Kast.** 1995. An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells. Identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301. Hum. Immunol. **44**:189-198.
33. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J. Virol. **74**:6800-6807.
34. **Voeten, J. T., G. F. Rimmelzwaan, N. J. Nieuwkoop, R. A. Fouchier, and A. D. Osterhaus.** 2001. Antigen processing for MHC class I restricted presentation of exogenous influenza A virus nucleoprotein by B-lymphoblastoid cells. Clin. Exp. Immunol. **125**:423-431.
35. **Weiner, A., A. L. Erickson, J. Kansopon, K. Crawford, E. Muchmore, A. L. Hughes, M. Houghton, and C. M. Walker.** 1995. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proc. Natl. Acad. Sci. U. S. A. **92**:2755-2759.

Chapter 5

Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes

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Submitted for publication

Abstract

In the present study, the recognition of epitope variants of influenza A viruses by human cytotoxic T lymphocytes (CTL) was investigated. To this end, human CD8+ CTL clones, specific for natural variants of the HLA-B*3501-restricted epitope in the nucleoprotein ($NP_{418-426}$), were generated. As determined in ^{51}Cr-release assays and by flow cytometry with HLA-B*3501-peptide tetrameric complexes, CTL clones were found to be specific for epitopes within one subtype or cross-reactive with heterosubtypic variants of the epitope. Using all eleven known natural variants of the epitope, positions in the 9-mer important for T cell recognition and involved in escape from CTL immunity were identified and visualized using multidimensional scaling. It was shown that positions 4 and 5 in the 9-mer epitope were important determinants of T cell specificity. The *in vivo* existence of CD8+ cells cross-reactive with homo- and heterosubtypic variants of the epitope was further confirmed using polyclonal T cell populations obtained after stimulation of PBMC with different influenza viruses. Based on the observed recognition patterns of the clonal and polyclonal T cell populations and serology it is hypothesized that consecutive infections with influenza viruses containing different variants of the epitope select for cross-reactive T cells *in vivo*.

Introduction

Cytotoxic T cells (CTL) play an important role in the control of viral infections. In influenza A virus-infected mice, CTL were shown to contribute to protective immunity against viruses of various subtypes (3, 12, 16, 30, 33), while human CTL have been reported to reduce morbidity (21, 23) and virus titers in the lung. Recently it was demonstrated that CTL immunity can exert selective pressure on influenza A virus CTL epitopes, further implicating a role for CTL in the control of influenza virus replication in infected individuals (5, 32). Besides amino acid changes in the anchor residues of CTL epitopes (32), changes in non-anchor residues were observed in an HLA-B*3501-restricted CTL epitope within the nucleoprotein (NP) of influenza A virus ($NP_{418\text{-}426}$) (5). Epitope variants of influenza A (H3N2) viruses (homosubtypic variants) were found to emerge in a chronological order (5). In addition, a variant of the epitope was identified in influenza A (H1N1) viruses, which differed from those found in A/H3N2 viruses (heterosubtypic variants). Although the majority of CTL epitopes are conserved between different subtypes (15, 16, 19, 35, 36), both human and murine CTL have been described that recognize a single subtype of influenza A virus (5, 6, 11, 17, 19). It has been shown that sequential infections with two influenza A viruses, containing different variants of the same epitope, induced CTL reactive against both epitopes, whereas infection with one influenza A virus only induced CTL reactive with the homologous peptide sequence (13, 29). These experiments were carried out in naïve mice and are difficult to extrapolate to humans. Furthermore, the data on the cross-reactive nature of human CTL is sparse.
The observed variability in the HLA-B*3501-restricted epitope $NP_{418\text{-}426}$ allows investigation of the cross-reactive nature of CTL obtained from humans, which constitute a natural host for influenza viruses. Here, the recognition of natural epitope variants by clonal and polyclonal T cell populations was studied. T cell recognition was studied in conventional ^{51}Cr-release assays, and also by direct visualization of T cells displaying monospecific or cross-reactive T cell receptors using HLA-B*3501-peptide tetrameric complexes (2).
It was found that most CTL recognize one subtype of currently circulating influenza A viruses. However, some of the CTL exhibited a broader recognition pattern and recognized homosubtypic and even heterosubtypic variants of epitope $NP_{418\text{-}426}$. Based on these findings obtained *in vitro*, the origin of these cross-reactive CTL is discussed.

Material and Methods

Cells

Peripheral blood mononuclear cells (PBMC) from normal healthy non-vaccinated HLA-B35+ blood donors (Tab. 1), between 30 and 48 years of age, were isolated between 1999 and 2001 using Lymphoprep® (Nycomed, Oslo, Norway) gradient centrifugation and cryopreserved at -135°C. HLA sero- and genotyping was performed in the laboratory for Histocompatibility and Immunogenetics at the

Bloodbank Rotterdam (Rotterdam, Netherlands) using a commercial typing system (GenoVision, Vienna, Austria).

Serology

Plasma samples were stored at –20°C and tested for the presence of influenza A virus (H3N2 and H1N1) specific antibodies in a hemagglutination inhibition (HAI) assay according to standard methods (22, 24) using turkey erythrocytes and four hemagglutinating units of virus. The sera were tested for antibodies against eleven vaccine strains of subtype H3N2 and six vaccine strains of the H1N1 subtype used since 1968. Ferret sera raised against the test antigens were used as positive controls. An HAI titer of >30 against a vaccine strain used in a particular season was considered indicative of an infection with an epidemic influenza A virus of that year.

Table 1: HLA-phenotype and age of blood donors

Donor	HLA-A and-B fenotype	Age (years)
1	A1,2; B57,35	44
2	A3,-; B27,35	46
3	A1,2; B7,35	41
4	A3,26; B27,35	30
5	A1,2; B8,35	37
6	A1,2; B8,35	48
7	A1,3; B8,35	38

Influenza A viruses and peptides

Sucrose gradient purified influenza A viruses were used for the infection of PBMC and cells of B-lymphoblastoid cell lines (BLCL). Resvir-9 is a reassortant virus between A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2), containing the NP, hemagglutinin (HA) and neuraminidase (NA) of A/Nanchang/933/95 with the LPFEKSTVM variant of the $NP_{418-426}$ epitope (designated NP_{ESV}), which was first identified in 1980 (Tab. 2). A second virus, A/Victoria/3/75 (H3N2) containing the LPFDKSTIM variant of the $NP_{418-426}$ epitope (designated NP_{DSI}), introduced in

Table 2: Name, sequence, virus and subtype of the different $NP_{418-426}$ epitopes

Virus Subtype	Virus strain	Epitope Name	Epitope sequence
H1N1	A/Netherlands/306/00	NP_{DTI}	LPFDKTTIM
H2N2/H3N2	-	NP_{DPI}	LPFDKPTIM
H3N2	A/Victoria/3/75	NP_{DSI}	LPFDKSTIM
H3N2	Resvir-9	NP_{ESV}	LPFEKSTVM

1972, was also used. As prototype of currently circulating H1N1 viruses in the Netherlands, we used A/Netherlands/306/00. This virus contains the LPFDKTTIM variant of the $NP_{418-426}$ epitope (designated NP_{DTI}, Tab. 2). The infectious virus titers of the virus preparations were determined in cell-culture using Madin-Darby-Canine-Kidney (MDCK) cells as indicator cells, as described previously (26). All peptide analogs of epitope $NP_{418-426}$ found in human influenza A viruses were manufactured, HPLC purified and analyzed by mass spectrometry (Eurogentec, Seraing, Belgium). Influenza A virus NP sequence information was obtained from the Influenza Sequence Database (Los Alamos National Laboratories, Los Alamos, N.M., USA) (20). Peptides were dissolved in DMSO at 5.0 mg/ml, diluted to 100 µM in RPMI 1640 (Invitrogen, Breda, Netherlands) and stored at -20°C until use.

In vitro stimulation of PBMC with influenza A virus
Stimulation of PBMC with influenza A virus was performed as previously described (4). The cells were resuspended at 1 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamin, 100 µg/ml streptomycin and 100 IU/ml penicillin (R10F). Following a one hour infection at 37°C with Resvir-9, A/Victoria/3/75 or A/Netherlands/306/00 at a multiplicity of infection (MOI) of three, the cells were washed once and resuspended in RPMI 1640 medium supplemented with 10% human AB serum, 2 mM glutamin, 100 µg/ml streptomycin, 100 IU/ml penicillin and 20 µM ß-mercaptoethanol (R10H) and added to uninfected PBMC at a ratio of 1:1 in a 25 cm^2 culture flask. The PBMC were incubated for two days before recombinant interleukin-2 (rIL-2, final concentration 50 U/ml, Chirion BV, Amsterdam, Netherlands) was added. Subsequently the cells were incubated for six days at 37°C and used as effector cells in a ^{51}Cr-release assay or stained with fluorescently-labeled HLA-B*3501-peptide tetrameric complexes (see below).

Generation of $NP_{418-426}$-specific CD8+ T cell clones
CTL clones were generated by limiting dilution (LDA) (32) after *in vitro* stimulation of PBMC infected with influenza A virus (Resvir-9) or pulsed with 10 µM of the variant $NP_{418-426}$ epitopes. After two subsequent rounds of non-specific stimulation with 1.0 µg/ml PHA (Sigma Aldrich, St. Louis, MD, USA), the ability of the cells to recognize the $NP_{418-426}$ epitope used during the initial stimulation was tested in an ELIspot assay (see below). The CTL clones with specificity for the $NP_{418-426}$ epitope were PHA stimulated in 75 cm^2 culture flasks prior to cryopreservation at -135°C. The CD8+, CD3+ cell phenotype of the $NP_{418-426}$-specific clones was confirmed by flow cytometry, using monoclonal antibodies (MAb) specific for CD3, CD4 and CD8 (Dako, Glostrup, Denmark).

Screening of virus- or epitope-specific T cell clones by ELIspot assay.
Approximately 10^4 cells of a T cell clone were added to an equal number of HLA-B*3501 positive BLCL cells that were infected with influenza A virus, uninfected or loaded with one of the $NP_{418\text{-}426}$ peptide variants, in 150 µl R10F in a 96-well V-bottom plate. Following a one-minute centrifugation at 140 x *g*, the plate was incubated for two hours at 37°C before the cells were transferred to a 96-well Silent Screen Plate (Nalge Nunc, Rochester, NY, USA) coated with anti-human IFN-γ MAb (Mabtech, Nacka, Sweden). After four hours at 37°C, the plates were washed and developed according to the manufacturer's recommendations. T cell clones that produced IFN-γ in the presence of peptide loaded BLCL or infected BLCL cells and failed to produce IFN-γ after stimulation with negative control BLCL cells, were selected for further investigation.

Table 3: CTL clone definition

CTL clone	Stimulation (conc.)	Influenza A virus subtype	Name	Donor
D1	LPFEKSTVM (10µM)	H3N2/1980	NP_{ESV}	5
C10	LPFEKSTVM (10µM)	H3N2/1980	NP_{ESV}	5
15.9	Infection with Resvir-9	H3N2/1980	NP_{ESV}	6
F10	LPFEKSTVM (10µM)	H3N2/1980	NP_{ESV}	7
G2	LPFEKSTVM (10µM)	H3N2/1980	NP_{ESV}	7
2384	LPFDKTTIM (10µM)	H1N1	NP_{DTI}	1
5017	LPFDKTTIM (10µM)	H1N1	NP_{DTI}	4
3180	LPFDKTTIM (10µM)	H1N1	NP_{DTI}	5
C4	LPFDKSTIM (10µM)	H3N2/1972	NP_{DSI}	5

Preparation of target cells for ^{51}Cr-release assay
BLCL were established as previously described (27), and used as target cells in ^{51}Cr-release assays. Cells of an HLA-B*3501 positive BLCL were infected with Resvir-9, A/Victoria/3/75 or A/Netherlands/306/00 at a MOI of three in RPMI medium, containing 0.1% bovine serum albumin, 2 mM glutamin, 100 µg/ml streptomycin and 100 IU/ml penicillin (R0.1B). After one hour at 37°C the cells were washed once in R10F and incubated in R10F for 16 h at 37°C. To test the ability of CTL clones to recognize synthetic peptides, 10^6 cells of a BLCL were loaded with 5 µM of different $NP_{418\text{-}426}$ variants for 16 hours at 37°C in R10F. Next, peptide loaded and virus-infected BLCL cells were incubated for 1 h at 37°C with $Na_2[^{51}Cr]O_4$, washed three times with R10F and used as target cells in a ^{51}Cr-release assay. As a measure of functional avidity of CTL (1), the minimal peptide concentration was determined for which 50% of the target cells were killed (EC_{50}). After a one-hour incubation of HLA-B*3501+ BLCL cells with $Na_2[^{51}Cr]O_4$ at 37°C, the cells were washed twice in R10F and distributed in 96-well V-bottom plates. Next, the cells were

subsequently incubated with ten-fold serial dilutions of peptide analogs of different $NP_{418-426}$ epitope variants in R10F for one hour at 37°C, washed once in R10F and used as target cells.

^{51}Cr-release assay

^{51}Cr-release assays (CTL assay) were performed as described previously (4). T cell clones were added to 5 x 10^3 ^{51}Cr-labeled target cells at different effector to target cell ratios (E:T-ratio). Following a 4-h incubation at 37°C, the culture 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)] x 100. The data are presented as the average percentage specific lysis of at least three wells.

Flow cytometry and tetramer staining

HLA-B*3501 molecules were complexed with four different HLA-B*3501-restricted $NP_{418-426}$ epitope variants within the NP of influenza A virus (NP_{ESV}, NP_{DSI}, NP_{DTI}, NP_{DPI}) as previously described (2). These natural variants of the $NP_{418-426}$ epitope were selected based on the predominance of these variants since 1957 in H2N2 (NP_{DPI}), since 1968 in H3N2 viruses (NP_{ESV}, NP_{DSI}, NP_{DPI}) and since 1977 in H1N1 viruses (NP_{DTI}). HLA-B*3501-peptide complexes were enzymatically biotinylated, FPLC purified and tetramerized by addition of allophycocyanin (APC)- or phycoerythrin (PE)-conjugated streptavidin (Sanquin Research at CLB, Amsterdam, Netherlands). Two or three-color fluorescence analysis was performed as described previously (31). Briefly, CTL clones (10^5) were stained in PBS supplemented with 2.0% FCS (P2F) and combinations of two HLA-B*3501-peptide tetrameric complexes with different $NP_{418-426}$ epitope variants. After staining for 20 minutes at 20°C, cells were washed with P2F and at least 10^4 events were acquired using a FACSCalibur flow cytometer (Becton Dickinson, Alphen a/d Rijn, Netherlands). Tetramer staining of influenza A virus stimulated PBMC was performed after 8-10 days of culture. Two hundred thousand cells were stained in P2F with combinations of two different HLA class I tetramers for 20 minutes at 20°C, followed by a 10 minute incubation with anti-CD8 MAb at 20°C. Cells were washed with P2F and on average 10^4 CD8+ cells were acquired. Data were analyzed using the software program CellQuest Pro (Becton Dickinson). A proportion tetramer positive cells in the CD8+ cells fraction of $\geq 0.4\%$ was considered positive.

Multidimensional scaling

An “antigenic map” based on the reactivity of the CTL clones against the variant peptides was constructed from the EC_{50} values of the variant $NP_{418-426}$ peptides for each CTL clone (18, 25). The map-making technique is a combination of metric (28) and non-metric (14) multidimensional scaling (MDS). The distance between two grids in the map corresponded to a ten-fold dilution of the peptide, starting at an EC_{50} value of 0.001 nM (the most parsimonious choice for these data). The distance between a peptide and a CTL clone or two peptides is a measure for respectively the

avidity between peptides and a CTL clone or antigenic dissimilarity between variant peptides. The clones and the peptides are positioned to minimize the sum of the squared errors between the target distances and the achieved map distances. The average error between target and achieved distances is 0.37 10-fold dilutions (SD = 0.45), indicating a good fit in two dimensions.

Results

Lytic activity of $NP_{418-426}$-specific CTL clones against target cells infected with various influenza A viruses

In addition to six previously described CTL clones ((5), Tab. 3), obtained after stimulation with two $NP_{418-426}$ epitope variants associated with the H3N2 subtype of influenza A viruses (NP_{ESV} and NP_{DSI}), three additional CTL clones were generated against the currently circulating $NP_{418-426}$ epitope variant of H1N1 viruses (NP_{DTI}). The original stimulation protocol, concentration of peptide, and origin of PBMC is given for each CTL clone (Tab. 3).
The ability of CTL clones to recognize influenza A virus-infected target cells was tested in a ^{51}Cr-release assay (Tab. 4). BLCL were infected with either an H1N1 virus (A/Netherlands/306/00; NP_{DTI}) or one of two H3N2 viruses (A/Victoria/3/75 or Resvir-9) and used as target cells. The latter two viruses contained either the NP_{DSI} or the NP_{ESV} variant of the $NP_{418-426}$ epitope respectively (Tab. 2). All CTL

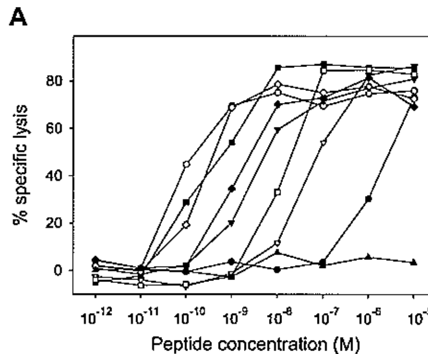
Table 4: Virus specificity of $NP_{418-426}$-specific CTL clones

CTL clone	CTL activity (average % lysis)[1]		
	Resvir-9 (NP_{ESV})	A/Victoria/3/75 (NP_{DSI})	A/Netherlands/306/00 (NP_{DTI})
D1	72	3	14
C10	73	0	6
15.9	63	12	3
F10	61	1	0
G2	91	14	26
2384	0	58	63
5017	2	60	74
3180	62	42	63
C4	0	53	44

[1] Average was calculated from two assays. The values represent the specific lysis minus the background lysis of uninfected target cells. The E:T ratio was 10:1.

clones exhibited lytic activity against at least one of the viruses (Tab. 4). CTL clones D1, C10, 15.9, F10 and G2 lysed target cells infected with Resvir-9, but not cells infected with A/Victoria/3/75 or A/Netherlands/306/00. In contrast, CTL clones 2384, 5017 and C4 recognized both the A/Netherlands/306/00 and A/Victoria/3/75

A



B Peptide concentration at which 50% of target cells are killed (EC_{50})

Peptide variants	CTL clones								
	H3N2/1980 (NP_{ESV})					H1N1 (NP_{DTI})			H3N2/1972 (NP_{DSI})
LPF---T-M[¶]	D1	C10	15.9	F10	G2	2384	5017	3180	C4
---DKT-I-	$>5x10^3$[#]	$>5x10^3$	$>5x10^3$	$>5x10^3$	$>5x10^3$	0.7	0.1	0.08	2
---DKP-I-	$>5x10^3$	$>5x10^3$	$>5x10^3$	$>5x10^3$	$>5x10^3$	$>10^4$	$>10^4$	26	0.8
---DKS-I-	18	17	$>10^4$	$>5x10^3$	$>5x10^3$	0.1	0.1	0.5	2
---DKS-V-	0.8	0.8	$>10^4$	12	$>5x10^3$	0.1	0.1	11	2
---DRT-I-	$>10^4$	$>10^4$	80	$>5x10^3$	100	$>5x10^3$	$>10^4$	1100	$>5x10^3$
---EKS-I-	0.8	1.0	0.1	8	0.8	$>10^4$	500	1.1	$>5x10^3$
---EKS-V-	0.2	0.8	0.1	0.03	0.1	$>5x10^3$	$>5x10^3$	0.5	$>5x10^3$
---EKT-I-	$>5x10^3$	$>5x10^3$	10	$>5x10^3$	8	$>5x10^3$	$>5x10^3$	3	$>5x10^3$

Figure 1: Functional avidity of $NP_{418-426}$-specific CTL clones
(A). Determination of EC_{50} values. The lytic activity of CTL clones was tested against 10-fold serial dilutions of nine different variants of the $NP_{418-426}$ epitope. The concentration (nM) at which 50% of the target cells (EC_{50}), at an E:T ratio of 10:1 were killed was determined. The results are shown for CTL clone 3180. Each symbol represents a different $NP_{418-426}$ epitope variant. (B) For all nine $NP_{418-426}$-specific CTL clones the EC_{50} values (nM) were determined of $NP_{418-426}$ epitope variants, which induced >25% specific lysis of target cells at a peptide concentration of 5 μM (data not shown). Absence of lytic activity at a concentration of 5 μM peptide is depicted as $>5x10^3$, while a value of $>10^4$ means that the EC_{50} value could not be determined, however target cell lysis >25% was observed at a concentration of 5 μM peptide. The CTL clones are divided into the three groups based on their original *in vitro* stimulus (table 3). [¶] Consensus sequence, [#] EC_{50} value in nM.

infected target cells but not the Resvir-9 infected cells. Finally, clone 3180 recognized all three virus-infected target cells to a similar extent, demonstrating the ability of this CTL clone to recognize homo- and heterosubtypic variants of the $NP_{418-426}$ epitope present in influenza A viruses of the H3N2 and H1N1 subtype.

Effect of amino acid changes at position 4, 5, 6 and 8 of the $NP_{418\text{-}426}$ epitope on the functional avidity of the CTL clones

In order to study the avidity of the respective T cell clones, target cells loaded with ten-fold serial dilutions of $NP_{418\text{-}426}$ 9-mers were used to determine the peptide concentration at which the T cell clones lysed 50% of the target cells (EC_{50}, see figure 1a). First, a pre-screening was performed with a high concentration of peptide (5 μM) to determine which epitopes were recognized by the different CTL clones (data not shown). Whenever a peptide induced more than 25% specific lysis, at a concentration of 5 μM, the EC_{50} value was determined for the clone/peptide combination. The EC_{50} values are depicted in the table in figure 1 and were used to assess the relative importance of the four amino acid positions (P4, P5, P6, P8) exhibiting variability in the $NP_{418\text{-}426}$ epitope. To exclude confounding effects of co-mutations, we preferred to consider single amino acid substitutions in the natural epitope variants, used to establish the CTL clones. If an amino acid substitution resulted in a greater than 100-fold change in EC_{50} value the amino acid mutation was considered to significantly alter T cell receptor avidity. The glutamic acid (E) to an aspartic acid (D) substitution at P4 or vice versa reduced T cell receptor avidity in six CTL clones (15.9, F10, G2, 2384, 5017 and C4), but not in clone 3180, D1 and C10. The effect of a substitution at P5 could only be studied in three CTL clones (2384, 5017, and 3180). The change of a lysine (K) to an arginine (R) at P5 reduced T cell receptor avidity in all three CTL clones, including the cross-reactive CTL clone 3180. At P6 either a serine (S) or a proline (P) was found in A/H3N2 viruses or a threonine (T) in A/H1N1 viruses. Clone C4, obtained after stimulation with peptide containing an S at P6, tolerated both the S→T and the S→P substitutions at P6. The CTL clones obtained after stimulation with peptide LPFDKTTIM containing the T at P6 (2384, 5017 and 3180) all tolerated the T→S mutation, but the T→P mutation reduced the avidity in clones 2384 and 5017 by more than 10000-fold, while the avidity of 3180 was reduced by a factor 300. To study the effect of the S→T substitutions at P6 on the recognition of $NP_{418\text{-}426}$ by NP_{ESV}-specific CTL clones, the EC_{50} values of LPFEKSTIM peptide and LPFEKTTIM peptide were compared for CTL clones that exhibited similar EC_{50} values for the peptides LPFEKSTVM and LPFEKSTIM peptide (D1, C10, 15.9 and G2). The S→T substitution at P6 significantly reduced TCR avidity in three of these four CTL clones. We therefore conclude that the effect of the S→T or T→S substitutions at P6 differs between the NP_{ESV}- and the $NP_{DTI/DSI}$-specific CTL clones.

The effect of amino acid substitutions at position 8 was evaluated for six CTL clones. CTL clones D1, C10, 15.9, G2 and C4 tolerated a valine (V) to isoleucine (I) substitution at P8, while the EC_{50} value of this variant epitope increased by a factor of 260 in CTL clone F10. We therefore conclude that the natural amino acids at P4 and P5 are important for CTL recognition, while the amino acids at P6 and especially P8 are less critical.

Antigenic map of T cell recognition constructed by multidimensional scaling

Using the EC_{50} values from the table in figure 1, an antigenic map was constructed by MDS. Using this mathematical procedure, the antigenic dissimilarity of the peptides and the avidity of $NP_{418\text{-}426}$-specific CTL clones for these peptides is

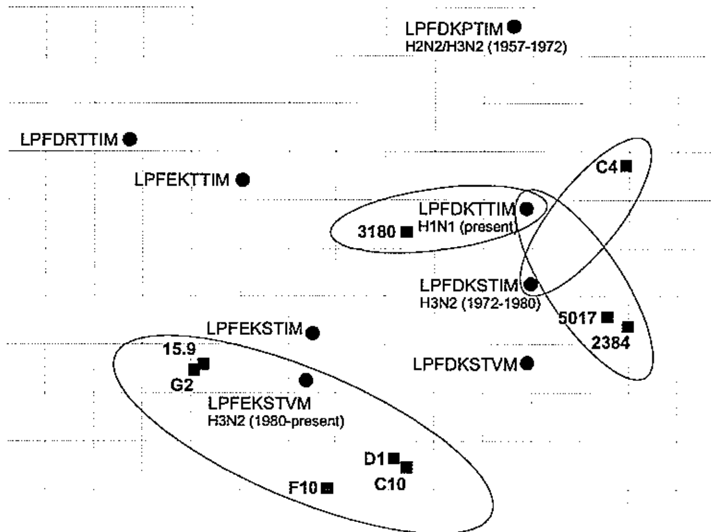


Figure 2: An "antigenic map" visualizing functional avidity between the variant $NP_{418-426}$ peptides and the CTL clones.
The functional avidity between the peptides and CTL clones was calculated from the EC_{50} values (table in figure 1). The CTL clones are depicted by squares while circles represent the variant peptides. The large circles contain the CTL clones and the $NP_{418-426}$ epitope variant used for *in vitro* stimulation of these clones. The distance between two grids corresponds to a 10-fold dilution in EC_{50} titer. For example, the EC_{50} value for clone 15.9 with peptide LPFEKSTIM is 0.1 nM, this is two 10-fold dilutions from the assumed baseline titer of 0.001 nM, thus the "target" distance from clone 15.9 to peptide LPFEKSTIM when constructing the map was two units.

visualized by the relative positioning of the peptides and T cell clones in a plot (figure 2). The large distance between peptides containing an E and those containing a D at P4 exemplified their antigenic dissimilarity. An R at P5 outplaced peptide LPFDRTTIM, confirming the importance of K at this position for recognition by the respective CTL clones. In addition, smaller antigenic distances were observed based on the observed amino acid variation at P6 and P8. The effect of amino acid substitutions at P6 on recognition by CTL differed between NP_{ESV}- and the $NP_{DSI/DTI}$-specific CTL. For the latter, the S→T or T→S substitution did not significantly alter the antigenicity of the peptides, whereas the S→T substitution altered the antigenicity of the peptide LPFEKSTIM for NP_{ESV}-specific clones as indicated by a significant increase of the antigenic distance in the plot (figure 2). The NP_{ESV}-specific T cell clones (D1, C10, 15.9, F10 and G2) were positioned in close proximity to the LPFEKSTVM peptide against which they were raised *in vitro*. Likewise, clones 2384, 5017 and C4 were positioned in proximity of their homologous peptides, although C4 was situated closer to the heterologous peptide

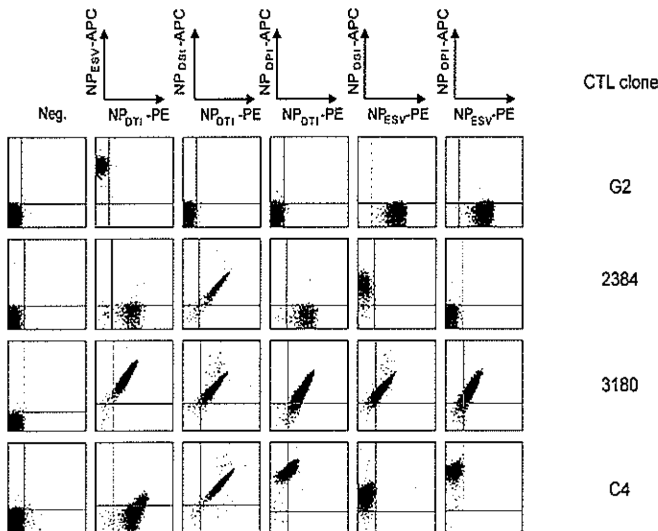


Figure 3: Binding of HLA-B*3501-peptide tetrameric complexes containing different $NP_{418-426}$ peptides to $NP_{418-426}$-specific CTL clones.
The $NP_{418-426}$-specific CTL clones were incubated with five different combinations of tetramers containing four different variants of the $NP_{418-426}$ epitope (NP_{ESV}, NP_{DSI}, NP_{DTI}, NP_{DPI}). NP_{DTI} tetramer is PE-labeled, NP_{DSI} and NP_{DPI} tetramers are APC-labeled and NP_{ESV} tetramers are available with PE and APC fluorescent label. Presented are four CTL clones with different reactivity patterns representative of the remaining five clones. CTL clone G2 bound to the different $NP_{418-426}$ peptide/HLA-B*3501 tetrameric complexes in the same way as CTL clones D1, C10, 15.9 and F10. CTL clone 2384 bound in a similar fashion as CTL clone 5017, while CTL clones 3180 and C4 were unique in their ability to bind to different $NP_{418-426}$ peptide/HLA-B*3501 tetrameric complexes.

LPFDKTTIM and clones 2384 and 5017 to the heterologous peptide LPFDKSTIM. Finally, clone 3180 was positioned in the middle of the plot, close to the H1N1 peptide and the H3N2 peptides from 1972 and 1980 confirming its ability to recognize hetero- and homosubtypic variants of the $NP_{418-426}$ epitope.

*Staining of CTL clones with HLA-B*3501-peptide tetrameric complexes containing different $NP_{418-426}$ variants*

The ability of CTL clones to recognize homo- and heterosubtypic variants of the $NP_{418-426}$ epitope was further studied using HLA-B*3501-peptide tetrameric complexes (tetramers) prepared with the four main variants of the $NP_{418-426}$ epitope (NP_{ESV}, NP_{DSI}, NP_{DTI}, NP_{DPI}) present in influenza A viruses of the H1N1 subtype (n = 1) and of the H3N2 subtype (n = 3, Tab. 2). The dotplots generated after flow-cytometry following incubation with differentially labeled tetramers are shown in figure 3. CTL clone G2 (figure 3) as well as CTL clones D1, C10, 15.9 and F10

(data not shown) bound to the NP_{ESV} tetramer, but did not react with any of the other tetramers, further demonstrating the failure of these T cell clones to recognize homo- and heterosubtypic variants of the $NP_{418-426}$ epitope. Of particular interest were CTL clones D1 and C10, which killed NP_{DSI} peptide-pulsed target cells at a relatively low EC_{50} value (18 nM and 17 nM respectively), but were unable to lyse A/Victoria/3/75 infected target cells (Tab. 4) and bind to NP_{DSI} containing tetramers. The lack of recognition of infected target cells coincided with the lack of binding to NP_{DSI} containing tetramers by CTL clones D1 and C10 indicating the fine specificity of these $NP_{418-426}$ tetramers. Clone 2384 (figure 3) and 5017 (data not shown) reacted with the tetramers containing the NP_{DTI} and NP_{DSI} peptide, but not with those containing the NP_{DPI} or the NP_{ESV} variant of the epitope. CTL clone C4 reacted similarly to clone 2384 and 5017, but also bound the NP_{DPI} tetramer (figure 3). Finally, clone 3180 bound all four tetramers containing different homo- and heterosubtypic variants of the epitope confirming the cross-reactive nature of this T cell clone. Overall, the ability of the CTL clones to bind tetramers with different peptides correlated well with their ability to kill virus-infected target cells.

*Staining of PBMC after in vitro stimulation with influenza A viruses using HLA-B*3501-peptide tetrameric complexes containing different $NP_{418-426}$ variants*

The specificity of $NP_{418-426}$ tetramer staining was confirmed in the previous section with well-defined CTL clones and this technology was also used to evaluate the specificity of $NP_{418-426}$-specific human polyclonal CTL responses using PBMC stimulated with influenza A virus. Following stimulation of PBMC of five HLA-B35+ donors with Resvir-9, a H3N2 virus containing the NP_{ESV} epitope, the percentage of NP_{ESV}-specific cells ranged from 1.1% to 18.5% of the CD8+ cells (figure 4a and data not shown). In the same PBMC cultures the percentage of NP_{DTI}-, NP_{DSI}- and NP_{DPI}-specific cells was also determined using HLA-B*3501-peptide tetrameric complexes containing the respective peptides. In the *in vitro* expanded PBMC of donor 1 small numbers of NP_{DTI} and NP_{ESV} double reactive CD8+ cells were observed (0.4%), demonstrating the presence of heterosubtypic reactive CD8+ cells (figure 4a). In the PBMC of the remaining four donors no NP_{DTI}-specific cells were detected after stimulation with Resvir-9. The ability to recognize homosubtypic variants of the $NP_{418-426}$ epitope following stimulation with a virus containing the NP_{ESV} variant peptide (Resvir-9) was variable between donors. In donors 1 and 2 a fraction of the NP_{ESV}-specific cells also bound to NP_{DSI} tetramers (figure 4a), while donor 3 to 5 did not (figure 4a and data not shown). Finally *in vitro* expanded NP_{ESV}-specific cells did not bind to the NP_{DPI} tetramer (figure 4a).
Similar experiments were performed with PBMC following stimulation with influenza viruses A/Victoria/3/75 (H3N2, NP_{DSI}) or A/Netherlands/306/00 (H1N1, NP_{DTI}). Of each of the five donors tested, 0.4%-4.5% of the CD8+ cells that were expanded after stimulation with influenza virus A/Victoria/3/75 bound to the homologous NP_{DSI} tetramers. In some donors a large fraction of the NP_{DSI}-specific cells also bound to the NP_{DTI} tetramers (donors 1, 3 and 4, figure 4b and data not shown), indicating a high degree of cross-reactivity between these epitopes.

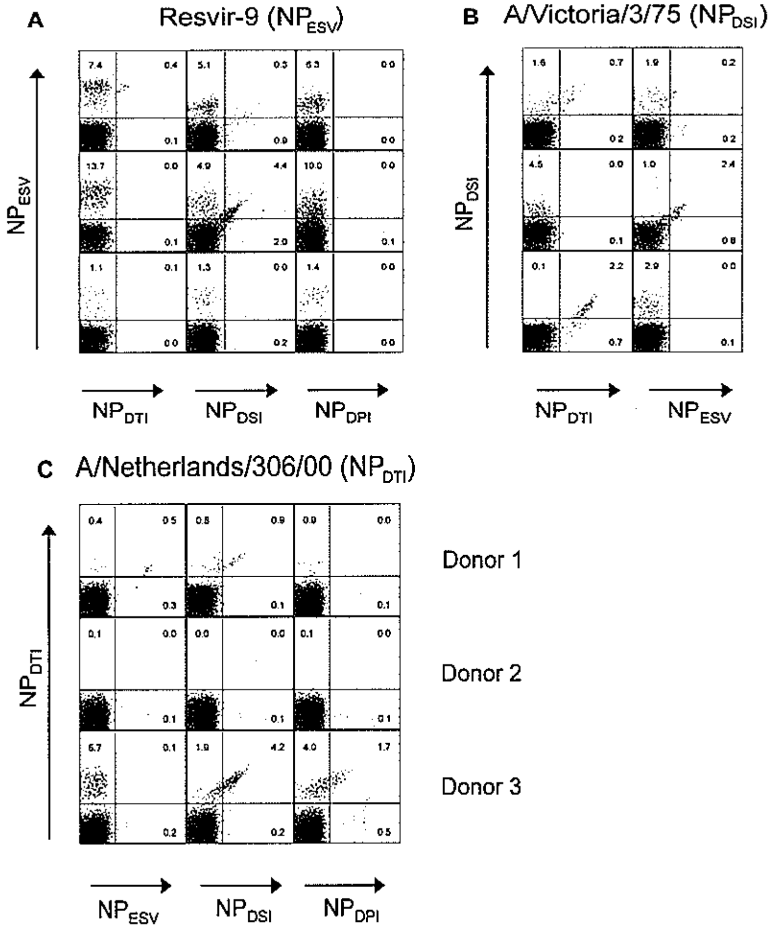


Figure 4: Staining of polyclonal $NP_{418\text{-}426}$-specific CTL response in PBMC stimulated with influenza A virus using HLA-B*3501-peptide tetrameric complexes.

PBMC of donors 1-5 were stimulated *in vitro* with Resvir-9 (NP_{ESV}), A/Victoria/3/75 (NP_{DSI}) or A/Netherlands/306/00 (NP_{DTI}) as indicated. The effector cells were incubated with APC- or PE-labeled NP_{ESV} and one of the three other tetramers (PE-labeled NP_{DTI}, APC-labeled NP_{DSI} or APC-labeled NP_{DPI}). Depicted are donors 1, 2 and 3 respectively stained with (A) NP_{ESV} tetramers (vertical) and NP_{DTI}, NP_{DSI}, or NP_{DPI} tetramers after stimulation with Resvir-9 or (B) NP_{DSI} tetramers (vertical) and NP_{DTI} or NP_{ESV} tetramers following stimulation with A/Victoria/3/75 or (C) NP_{DTI} tetramers (vertical) and NP_{ESV}, NP_{DSI} or NP_{DPI} tetramers following stimulation with A/Netherlands/306/00. Staining profiles of donors 4 and 5 were not shown, since they resembled those of donor 3 (donor 4) or had low to undetectable levels of tetramer positive cells. The percentages indicate the percentage tetramer positive cells in 10^4 CD8+ cells.

The NP_{DSI}-specific cells from donor 2 however, cross-reacted with the NP_{ESV} tetramer and not with the NP_{DTI} tetramer, confirming the existence of homosubtypic cross-reactive CTL in this donor.
Upon stimulation with influenza virus A/Netherlands/306/00, NP_{DTI}-specific CD8+ cells were detected in some PBMC cultures (range 0.0%-6.2%, figure 4c). Most of the NP_{DTI}-specific CD8+ cells also bound the NP_{DSI} tetramers (donor 1, 3 and 4, figure 4c and data not shown). In addition, a portion of the NP_{DTI}-specific cells from donor 1 cross-reacted with NP_{ESV} tetramers (figure 4c), confirming the existence of these cells following stimulation with Resvir-9 virus. Finally, only in donor 3 some NP_{DTI}-specific cells in the PBMC cultures stimulated with A/Netherlands/306/00 also bound to the NP_{DPI} tetramers (figure 4c).
Thus, tetramer staining provided evidence for the existence of CTL cross-reactive with current H3N2 (NP_{ESV}) and H1N1 (NP_{DTI}) viruses in one donor, whereas in the other four donors only influenza A virus subtype-specific cells were demonstrated or cells cross-reactive with previous H3N2 (NP_{DSI}) and current H1N1 (NP_{DTI}) viruses.

Influenza A virus-specific antibody titers
To correlate the $NP_{418-426}$-specific CTL response in the PBMC of donors to the history of influenza A virus infections, serum HAI titers specific for A/H1N1 and A/H3N2 viruses were determined.
Serum antibodies were detected in donor 1 against the 1978-vaccine strain (H1N1), which cross-reacted with later vaccine strains, and the 1991 vaccine strain. Antibodies specific for H3N2 viruses were observed against A/Hong Kong/1/68 (NP_{DPI}) and the 1995 vaccine strain (NP_{ESV}). These findings indicate that donor 1 was exposed to influenza A viruses of the H1N1 subtype around 1978 and 1991 and to the H3N2 viruses around 1968 and 1995 (figure 5). In donor 2 antibodies specific for 1968, 1977, and 1995 vaccine strains of the H3N2 subtype were readily detected (figure 5). These findings suggest that this donor has experienced multiple exposures to influenza A viruses of the H3N2 subtype but not to those of the H1N1 subtype. Donor 3 had serum antibodies against the 1968 and 1995 strain of the H3N2 subtype (figure 5). Donor 4 was found to have serum antibodies to viruses of the A/H3N2 (1977, 1979, 1982, 1987 and 1995) and the A/H1N1 subtype (1978, 1983 and 1996), whereas in donor 5 solely antibodies against viruses of the H3N2-subtype from 1977, 1979, 1995 and 1998 were detected (figure 5).

Discussion

In the present study the recognition of natural variants of an HLA-B*3501-restricted CTL epitope within the NP of influenza A viruses by human clonal and polyclonal CTL populations was investigated. Using classical ^{51}Cr-release assays and direct visualization of specific T cells with tetramers, CTL were identified that were specific for a single subtype variant or cross-reactive with homo- and heterosubtypic variants. The use of CTL clones specific for the respective epitope variants allowed the elucidation of the molecular basis for the recognition of these variants. Based on

the finding that cross-reactive CTL were found at low frequencies in PBMC stimulated with influenza virus, we hypothesize that in response to influenza virus infections, HLA-B*3501+ individuals develop a CTL response against the homologous epitope variant of which a small proportion may be cross-reactive with other variants. These cross-reactive cells may then be further expanded upon a subsequent infection with viruses carrying a heterologous variant epitope. It was assumed that the study subjects were infected in the past with a homogeneous virus population with regard to the epitope $NP_{418\text{-}426}$, based on epidemiological data on the circulation of these variants (20).

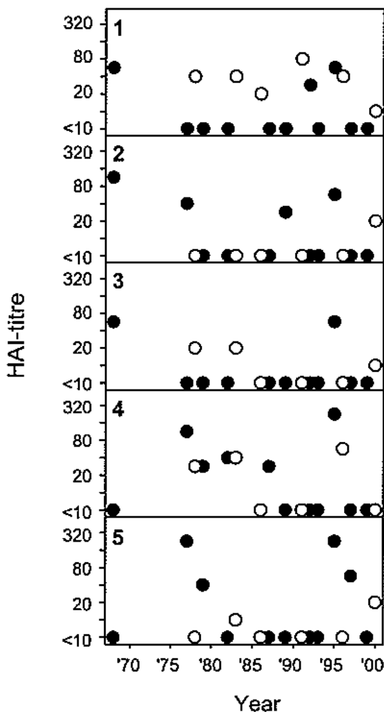


Figure 5: Influenza A virus-specific HAI titers in plasma of HLA-B35+ blood donors
HAI assays were performed on plasma obtained from donors 1-5, using six different H1N1 vaccine strains (○) and eleven different H3N2 vaccine strains (●) used since 1968. The HAI titers specific for each vaccine strain are depicted for donors 1-5 against the years in which the vaccine strains were isolated.

The results obtained in ^{51}Cr-release assays with virus-infected target cells and direct visualization with labeled tetramers correlated well, confirming the specificity of these procedures. When target cells were used in the ^{51}Cr-release assay pulsed with peptides some discrepant results were obtained, in particular with CTL cell clones D1 and C10, which were able to recognize the NP_{ESV} and the NP_{DSI} peptide at very low EC_{50} values. These clones however, failed to bind NP_{DSI} tetramers or recognize target cells infected with influenza virus A/Victoria/3/75. Using serial dilutions of the peptides, the avidity of the CTL clones for the peptides was determined in ^{51}Cr-release assays and the effect of the amino acid substitutions was assessed. *In vitro* three T cell clones were established after stimulation with peptide NP_{DTI} (5017, 2384 and 3180) and one after stimulation with NP_{DSI} (C4). Although these clones had high avidity for these peptides it is not clear which variant of the epitope had primed

these CTL *in vivo*. As can be judged from the EC_{50} values (table in figure 1) and the MDS plot (figure 2), some clones exhibited the highest avidity for a heterologous peptide. For example, clone C4 may have been directed to NP_{DPI} and clone 2384 to NP_{DSI}. In contrast, T cell clones raised *in vitro* to peptide NP_{ESV} all showed the highest avidity to this peptide, suggesting that indeed it was the homologous peptide responsible for the activation of these cells *in vivo*.

The $NP_{418-426}$ epitope was found to vary in influenza viruses isolated during the past 70 years at position 4, 5, 6 and 8 of the 9-mer epitope. All peptides retained the anchor residues at position 2 and 9 indicating that they bind to HLA-B*3501 with equal affinity, as has been demonstrated previously in an HLA-peptide binding assay (5). Substitutions at P4 and P5 were found to have the largest effect on recognition by specific T cells as can be judged from the EC_{50} values and therefore the antigenic distance in the MDS plot. The $D_{421}E$ substitution in 1980 allowed the virus to escape from NP_{DSI} specific CTL (5). These findings are in agreements with data obtained with dengue virus-specific CTL, confirming the importance of position 4 for the recognition of an HLA-B*3501-restricted epitope (34). Although a mutation at P4 had a major impact on TCR recognition, CTL do exist (3180) that recognize peptides containing either an E or a D at position 4. These CTL may have been selected for by repeated infections with different influenza A viruses containing different variants of the epitope. The $K_{422}R$ substitution also had a dramatic effect on the recognition of the epitope. However the R_{422} sequence is rare and has only been found in influenza A viruses isolated in 1933 and 1934, and in several human isolates of avian origin. Substitutions at P6 and P8 had relatively little impact on TCR recognition, although for most NP_{ESV}-specific T cell clones the T→S substitution at P6 reduced the TCR avidity for the epitope. In contrast to $NP_{418-426}$-specific CTL, CTL specific for an HLA-B*3501-restricted epitope of dengue virus ($NS_{500-508}$) tolerated substitution at position 8 poorly (34).

Upon stimulation of PBMC with different influenza A viruses, various recognition patterns were observed that correlated partially with the history of infections of the respective donors (see below). In three donors (no. 1, 3 and 4), CTL expanded after stimulation with influenza virus A/Netherlands/306/00 (of the H1N1 subtype containing NP_{DTI}) were cross-reactive with the NP_{DSI} variant present in influenza viruses of the H3N2 subtype, circulating between 1972 and 1980, and vice versa. Thus also stimulation with A/Victoria/3/75 (H3N2) lead to the expansion of NP_{DSI} specific cells cross-reactive with NP_{DTI}. This finding resembles the cross-reactivity observed with CTL specific for epitopes in HIV-1, dengue virus and SIV (8, 9, 34). HIV-1-specific CTL could recognize variants of epitopes present in different clades of this virus. An important difference between influenza virus and HIV-1 infections is that individuals usually are not infected with HIV from different clades whereas for influenza, repeated infections with variant viruses of distinct or the same subtype is very common. For that reason, repeated infections with variant viruses may select for cross-reactive CTL from the pool of $NP_{418-426}$-specific CTL induced during the primary infection. To correlate the presence of cross-reactive CTL with the history of influenza A virus infections of the respective donors, serology was performed. Donor 1 was infected with an influenza A virus of the H1N1 subtype and subsequently with an H3N2 virus. This is in agreement with the observation in this

donor of NP_{DTI}- and NP_{ESV}-specific cells. A small proportion of these cells cross-reacted with NP_{ESV} and NP_{DTI}. Similarly, donor 2 and 5 were found to be exposed to viruses of the H3N2 subtype circulating in 1977 and 1995 and not to H1N1 viruses. As a result, NP_{ESV} mono-specific CTL were found in both donors and not T cells reactive with the NP_{DTI} (H1N1) variant of the epitope. In addition homosubtypic cross-reactive CTL were found in donor 2. In donor 4 the serology correlated with the presence of NP_{ESV}- and NP_{DTI}-specific cells, although cross-reactive cells were not observed in this donor. A similar reaction pattern was observed in donor 3, although H1N1-specific antibody titers were very low. This and the absence of heterosubtypic cross-reactive CTL may be explained by the long period between infection with an H1N1 virus and an H3N2 virus. Thus in two out of five donors tested, there was evidence for the existence of T cells cross-reactive with homo- and heterosubtypic variants of the $NP_{418-426}$ epitope. The heterogeneity in the $NP_{418-426}$-specific responses in these donors of similar age can be the result of differences in history of infection, as well as many other factors influencing the CTL response in these donors.
In mice it has been demonstrated *ex vivo* (13) and *in vitro* (29) that consecutive infections with influenza viruses containing different variants of the same epitope ($NP_{366-374}$) resulted in the expansion of CTL able to recognize both variants of the epitope, whereas infection with the same virus resulted in the induction of mono-specific cells. This expansion of cross-reactive CTL may even take place after consecutive infections with unrelated viruses as has been observed in mice (7, 10). We hypothesize that also in humans cross-reactive CTL can be selected for against variable epitopes by multiple infections with influenza viruses carrying variant epitopes (apart from true cross-reactive responses against conserved epitopes).
The percentage of heterosubtypic cross-reactive T cells specific for the $NP_{418-426}$ epitope is relatively small in the subjects compared to those observed *in vivo* in mice. In some of the donors there was no evidence for exposure to both subtypes, explaining the absence of cross-reactive CTL. Secondly, the timing of the infections may be crucial as the frequency of cross-reactive CTL in the pool of memory cells may have declined to undetectable levels, unable to dominate the response to the variant epitope.
We conclude that upon influenza virus infections a small proportion of the $NP_{418-426}$-specific CTL can cross-react with variants of the epitope. Apparently the flexibility of the TCR of this subset of CTL allows the recognition of naturally occurring variants of the epitope which may be escape mutants selected for by CTL-mediated immune pressure. This subset of cross-reactive CTL may increase after repeated infections with heterologous viruses and may contribute to protective immunity against arising mutant viruses.

References

1. **Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky.** 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc. Natl. Acad. Sci. U. S. A. **93:**4102-7.
2. **Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science. **274:**94-6.
3. **Bender, B. S., T. Croghan, L. Zhang, and P. A. Small.** 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. **175:**1143-1145.
4. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. The magnitude and specificity of influenza A virus-specific cytotoxic T lymphocyte responses in humans is related to HLA-A and -B phenotype. J. Virol. **76:**582-90.
5. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. J. Virol. **76:**2567-72.
6. **Braciale, T. J., M. E. Andrew, and V. L. Braciale.** 1981. Heterogeneity and specificity of cloned lines of influenza-virus specific cytotoxic T lymphocytes. J. Exp. Med. **153:**910-23.
7. **Brehm, M. A., A. K. Pinto, K. A. Daniels, J. P. Schneck, R. M. Welsh, and L. K. Selin.** 2002. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. Nat. Immunol. **3:**627-34.
8. **Buseyne, F., M. L. Chaix, C. Rouzioux, S. Blanche, and Y. Riviere.** 2001. Patient-specific cytotoxic T lymphocyte cross-recognition of naturally occurring variants of a human immunodeficiency virus type 1 (HIV-1) p24gag epitope by HIV-1-infected children. J. Virol. **75:**4941-6.
9. **Cao, H., P. Kanki, J. L. Sankale, A. Dieng-Sarr, G. P. Mazzara, S. A. Kalams, B. Korber, S. Mboup, and B. D. Walker.** 1997. Cytotoxic T lymphocyte cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development. J. Virol. **71:**8615-23.
10. **Chen, H. D., A. E. Fraire, I. Joris, M. A. Brehm, R. M. Welsh, and L. K. Selin.** 2001. Memory CD8+ T cells in heterologous antiviral immunity and immunopathology in the lung. Nat. Immunol. **2:**1067-76.
11. **Effros, R. B., P. C. Doherty, W. Gerhard, and J. Bennink.** 1977. Generation of both cross-reactive and virus-specific T cell populations after immunization with serologically distinct influenza A viruses. J. Exp. Med. **145:**557-68.
12. **Fu, T. M., L. Guan, A. Friedman, T. L. Schofield, J. B. Ulmer, M. A. Liu, and J. J. Donnelly.** 1999. Dose dependence of CTL precursor frequency

induced by a DNA vaccine and correlation with protective immunity against influenza virus challenge. J. Immunol. **162:**4163-4170.
13. **Haanen, J. B., M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher.** 1999. Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. J. Exp. Med. **190:**1319-1328.
14. **Kruskal, J.** 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika. **29:**1-27.
15. **Kuwano, K., V. E. Reyes, R. E. Humphreys, and F. A. Ennis.** 1991. Recognition of disparate HA and NS1 peptides by an H-2Kd-restricted, influenza specific CTL clone. Mol. Immunol. **28:**1-7.
16. **Kuwano, K., M. Tamura, and F. A. Ennis.** 1990. Cross-reactive protection against influenza A virus infections by an NS1-specific CTL clone. Virology. **178:**174-9.
17. **Lamb, J. R., D. D. Eckels, M. Phelan, P. Lake, and J. N. Woody.** 1982. Antigen-specific human T lymphocyte clones: viral antigen specificity of influenza virus-immune clones. J. Immunol. **128:**1428-32.
18. **Lapedes, A., and R. Farber.** 2001. The geometry of shape space: application to influenza. J. Theor. Biol. **212:**57-69.
19. **Lukacher, A. E., V. L. Braciale, and T. J. Braciale.** 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. J. Exp. Med. **160:**814-26.
20. **Macken, C., H. Lu, J. Goodman, and L. Boykin** 2001. The value of a database in surveillance and vaccine selection. Elsevier Science, Amsterdam.
21. **Mackenzie, C. D., P. M. Taylor, and B. A. Askonas.** 1989. Rapid recovery of lung histology correlates with clearance of influenza virus by specific CD8+ cytotoxic T cells. Immunology. **67:**375-81.
22. **Masurel, N., P. Ophof, and P. de Jong.** 1981. Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. J. Hyg. (Lond). **87:**201-9.
23. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare.** 1983. Cytotoxic T cell immunity to influenza. N. Engl. J. Med. **309:**13-7.
24. **Palmer, D., W. Dowdle, M. Coleman, and G. Schild** 1975. Haemagglutination inhibition test, p. 25-62. *In* Anonymous (ed.), Advanced laboratory techniques for influenza diagnosis. Procedural Guide. US Dept. Hlth. ED., Atlanta.
25. **Perelson, A. S., and G. F. Oster.** 1979. Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination. J. Theor. Biol. **81:**645-70.
26. **Rimmelzwaan, G. F., M. Baars, E. C. Claas, and A. D. Osterhaus.** 1998. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J. Virol. Methods. **74:**57-66.
27. **Rimmelzwaan, G. F., N. Nieuwkoop, A. Brandenburg, G. Sutter, W. E. Beyer, D. Maher, J. Bates, and A. D. Osterhaus.** 2000. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune

responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine. **19:**1180-7.

28. **Shepherd, R.** 1963. Analysis of proximities as a technique for the study of information processing in man. Human Factors. **5:**33-48.

29. **Townsend, A. R., and J. J. Skehel.** 1984. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. J. Exp. Med. **160:**552-63.

30. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, and et al.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. **259:**1745-9.

31. **van Baarle, D., E. Hovenkamp, M. F. Callan, K. C. Wolthers, S. Kostense, L. C. Tan, H. G. Niesters, A. D. Osterhaus, A. J. McMichael, M. H. van Oers, and F. Miedema.** 2001. Dysfunctional Epstein-Barr virus (EBV)-specific CD8(+) T lymphocytes and increased EBV load in HIV-1 infected individuals progressing to AIDS-related non-Hodgkin lymphoma. Blood. **98:**146-55.

32. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J. Virol. **74:**6800-6807.

33. **Yap, K. L., G. L. Ada, and I. F. McKenzie.** 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. Nature. **273:**238-239.

34. **Zivny, J., I. Kurane, A. M. Leporati, M. Ibe, M. Takiguchi, L. L. Zeng, M. A. Brinton, and F. A. Ennis.** 1995. A single nine-amino acid peptide induces virus-specific, CD8+ human cytotoxic T lymphocyte clones of heterogeneous serotype specificities. J. Exp. Med. **182:**853-63.

35. **Zweerink, H. J., B. A. Askonas, D. Millican, S. A. Courtneidge, and J. J. Skehel.** 1977. Cytotoxic T cells to type A influenza virus; viral hemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. Eur. J. Immunol. **7:**630-5.

36. **Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, M. J. Crumpton, and B. A. Askonas.** 1977. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. Nature. **267:**354-6.

Chapter 6

Influenza A virus-specific T cell immunity in humans during aging

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Virology 2002;299:100-108

Abstract

To study the decreasing responsiveness of the immune system during aging, influenza virus-specific cellular immunity was investigated in a cohort of healthy blood donors between 18 and 70 years of age. The percentage of influenza A virus-specific T cells was determined by flow cytometry and found not to change during aging. After stimulation with phorbol 12-myristate 13-acetate and ionomycin, an increase in the percentage of IFN-γ and IL-4 producing CD8+ T cells was observed during aging. In addition, the cytotoxic T lymphocyte (CTL) activity was investigated in two additional groups of five donors, 18-20 and 68-70 years of age. The lytic capacity of purified CD8+ T cells, after *in vitro* stimulation of peripheral blood mononuclear cells with influenza A virus, seemed lower in 68- to 70-year-old donors than in 18- to 20-year-old donors. Therefore we conclude that the reduced CTL activity in the elderly is not the result of a lower frequency of virus-specific T cells, but more likely the result of impaired antigen-specific proliferation or lower lytic capacity of these cells.

Introduction

The elderly form a growing group at high risk for complications related to infections with respiratory syncitial virus (RSV) and influenza virus, resulting in high morbidity and mortality rates (2, 15, 16, 19, 29). The more severe clinical course of these virus infections in the elderly can partially be attributed to a reduced responsiveness of the immune system, also known as immunosenescence. Immunosenescence has been associated with reduced T cell proliferative responses to antigens and mitogens, reduced interleukin-2 production, increased interferon-γ (IFN-γ) production and reduced expression of the co-stimulatory molecule CD28 on T cells (18, 28, 34, 42). In the elderly this phenomenon may also underlie lower vaccine efficacy (12).
In addition to the induction of virus-specific antibodies, the induction of cellular immune responses has been shown to contribute to protective immunity against influenza. In mouse models the protective effect of CD8+ cytotoxic T lymphocytes (CTL) has been demonstrated against homologous and heterologous subtypes of influenza virus (17, 23, 40, 44). Also in humans protective immunity was associated with the induction of CTL responses (4, 27). Furthermore, the selective pressure of CTL-mediated immunity indicates a role for CTL in the control of influenza virus replication in infected individuals (7, 41). It has been described that CTL activity is reduced in the elderly both *in vivo* (18) and *in vitro* (25, 26, 35). Several steps determine the extent of CTL activity after stimulation with antigen during infection. These steps include the virus-specific CTL precursor frequency (CTLp) in the pool of memory cells, the stimulation and subsequent expansion of virus-specific cells and the cytolytic effector function per cell. The latter is still a matter of debate since some reports describe a reduced expression of perforin and granzyme in the elderly, while others describe no change in CTL activity on a per cell basis (18). Currently no data are available on the frequency of virus-specific CTL during aging. In the present study, the CTLp frequency of influenza virus-specific CTL was investigated in a cohort of 87 donors of various ages, ranging from 18 to 70 years. It was found that the number of virus-specific CTL remained stable during aging. Thus the reduced *in vitro* CTL activity of activated peripheral blood mononuclear cells (PBMC) obtained from elderly people cannot be explained by fluctuations in the quantity of virus-specific CTL. Functional studies performed with PBMC from a group of 18- to 20-year-old individuals and a group of 68- to 70-year-old individuals indicated that the observed reduction of CTL activity *in vitro* is more likely the result of a reduced proliferation of T cells upon activation or a reduced lytic capacity of the CTL.

Material and Methods

Human subjects
Blood samples of 10 ml were collected in heparin-containing vacutainers (Becton Dickinson, Plymouth, UK) at the bloodbank in Rotterdam from 87 donors of various ages, ranging from 18 to 70 years (Tab. 1). Within 4 h after collection, the blood was

diluted in PBS (1:1), and the PBMC were isolated by gradient centrifugation using Lymphoprep® (Nycomed, Oslo, Norway), as recommended by the manufacturer. The cells were washed three times in PBS and left overnight at 37°C in RPMI 1640 (Life Technologies, Rockville, USA) supplemented with 10% pooled human AB serum, streptomycin (100 U/ml), penicillin (100 μg/ml), glutamin (2 mM) and 2-mercaptoethanol (2 μM) (R10H). The plasma, 1:1 diluted in PBS, was collected and stored at – 20°C. From two groups of donors, aged 18-20 year (n = 5) and 68-70 year (n = 5), buffy-coats were obtained from which PBMC were isolated and cryopreserved as previously described (6). Before collecting blood, informed consent was obtained from each of the blood donors.

Table 1: Demographics of study participants

Age group	Total no.	Female	Male
18-25	10	5 (50%)[a]	2 (20%)[a]
26-35	12	7 (58%)	5 (42%)
36-45	14	8 (57%)	6 (43%)
46-55	22	8 (36%)	14 (64%)
56-65	23	7 (30%)	16 (70%)
66-70	6	1 (17%)	5 (83%)
Total	87	36 (41%)	48 (55%)

[a] The gender of three donors was unknown

Preparation of B lymphoblastoid cell lines (BLCL)
Autologous Epstein-Barr virus (EBV)-transformed BLCL of each of the five young and five old donors was established by culturing 1-5 x 10^6 PBMC in 1.0 ml culture supernatant from the EBV producing cell line S594 in 24-wells plates as previously described (37).

Influenza virus
Sucrose-gradient purified influenza A virus (H3N2) Resvir-9, a reassortant between A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2), containing the hemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) of A/Nanchang/933/95, was used for the infection of PBMC or BLCL. The infectious virus titer (1 x 10^9 $TCID_{50}$) was determined in cell culture using Madin-Darby-Canine-Kidney (MDCK) cells as indicator cells as previously described (36). For lymphocyte proliferation studies, Influvac 97/98 (kindly provided by Solvay Pharmaceuticals BV, Weesp, Netherlands), a trivalent vaccine consisting of the influenza virus glycoproteins HA and NA from A/Johannesburg/82/95 (H1N1), A/Nanchang/933/95 (H3N2) and B/Harbin/7/94, was used.

Enumeration of influenza A virus-specific T cells

PBMC were resuspended and washed twice in RPMI 1640 supplemented with 10% fetal calf serum (FCS), streptomycin (100 U/ml), penicillin (100 μg/ml) and glutamin (2 mM) (R10F) to remove influenza virus-specific antibodies present in the human serum. Subsequently, 1 x 10^6 PBMC were infected for 1 h at 37°C with Resvir-9 at a multiplicity of infection (MOI) of 3. Uninfected PBMC were included as a control. After 1 h incubation at 37°C, the PBMC were spun down, resuspended in R10H and divided over two wells in a 96-well round-bottom Costar® plate (Corning Incorporated, Corning, NY, USA), with each well containing 3 x 10^5 PBMC in 150 μl R10H. After a previously determined optimal period of 5 h at 37°C, GolgiStop (Pharmingen, San Diego, CA, USA) was added and left for a subsequent six hours at 37°C. Influenza virus-specific T cells were visualized by intracellular IFN-γ staining and flow cytometry (see below).

Enumeration of IFN-γ and IL-4 producing T cells upon non-specific stimulation

PBMC, 2 x 10^5 cells per well, were stimulated with 50 ng/ml PMA (Sigma Chemical Co., St Louis, MO, USA) and 500 ng/ml ionomycin (Sigma) for six hours at 37°C in R10F. The percentage IFN-γ and interleukin-4 (IL-4) positive cells were determined using an intracellular cytokine staining protocol and flow cytometry (see below).

Intracellular cytokine staining and flow cytometry analysis

After stimulation of PBMC with PMA/ionomycin or influenza A virus, the cells were transferred to a V-bottom shaped 96 wells plate and subsequently stained, fixed and permeabilized with the Cytofix/Cytoperm kit (Pharmingen) according to instructions provided by the manufacturer. Briefly, PBMC were washed once in PBS with 2.0% FCS (P2F) and incubated with a mix of anti-human CD3-RPE-Cy5 (Dako, Glostrup, Denmark) and anti-human CD8-FITC (Dako) or anti-human CD3-RPE-Cy5 and anti-human CD4-FITC (Dako) in 50 μl P2F for 30 min at 4°C. Following two washing steps with P2F, the cells were fixed with 100 μl Cytofix buffer for 30 min at 4°C. Next, the cells were washed twice in permeabilization buffer and subsequently incubated for 30 min at 4°C in 50 μl permeabilization buffer containing anti-human IFN-γ-PE (Pharmingen) or anti-human IL-4-PE (Pharmingen). After two washing steps with permeabilization buffer, the cells were resuspended in P2F, and the number of cytokine positive cells was determined using flow cytometry (FACS scan, Becton Dickinson). The percentage cytokine positive cells within the CD4+ or CD8+ T cell fraction was obtained from one well, while percentage cytokine positive cells within the CD3+ T cells were calculated from duplicate wells. Sporadically we were unable to determine the virus-specific precursor frequency. After stimulation with influenza virus, the percentage IFN-γ+ cells was determined within the CD3+, CD4+ and CD8+ T cell compartments (figure 1a and 1b), while after mitogenic stimulation, both the percentage IFN-γ+ and the percentage IL-4+ cells were determined within CD8+ T cell population and the percentage IFN-γ+ cells were determined within CD3+ cells. Results are given

as the percentage cytokine-positive cells, calculated from the percentage after stimulation with either influenza virus or mitogen minus unstimulated control cells. The cutoff value was calculated from the average percentage cytokine positive cells of the unstimulated PBMC plus one time the standard deviation (SD).

Serology

Serum samples were tested for the presence of influenza A virus-specific antibodies in the HI test according to standard methods (24, 33) using turkey erythrocytes and four hemagglutinating units of virus. The samples were tested against 10 influenza A virus (H3N2) vaccine strains from the introduction of H3N2 (A/HongKong/1/68) till one of the most recent H3N2 vaccine strain (A/Sydney/5/97) and for antibodies against 9 influenza A virus (H1N1) strains, from the first isolate of H1N1 (A/Puerto Rico/8/34) till the latest H1N1 vaccine component (A/New Caledonia/20/99). Ferret sera raised against the test antigens were used as positive controls.

Phenotypic analysis of PBMC

Cryopreserved PBMC, from the groups of 18- to 20-year-old and 68- to 70-year-old donors (n = 10), were analyzed for memory and naïve T cell phenotype and CD28 expression. Approximately 2 x 10^5 PBMC were resuspended in 50 μl P2F and incubated for 30 min at 4°C with different combinations of MAb. For the staining of T lymphocytes MAb directed to human CD3 (Dako), CD4 (Dako), CD8 (Dako), CD45RA-FITC (Pharmingen), CD45RO-Cy-Chrome (Pharmingen) and CD28-RPE (Pharmingen) were used. The cells were subsequently washed once in P2F and analyzed by flow cytometry.

Proliferative response of donor PBMC

To determine their proliferative capacity, PBMC (1 x 10^5) of donors between 18 and 20 or 68 and 70 years of age were transferred to a U-bottom shaped 96 wells plate in 150 μl R10H and stimulated with a twofold serial dilution of Influvac 97/98, starting at 2.0 μg/ml HA per virus strain. A medium control was included to determine nonspecific proliferation. After a five-day incubation at 37°C, 0.5 μCi [^{3}H]-thymidine was added to each well and subsequently incubated for 18 hours at 37°C prior to harvesting. Results were calculated from triplicate wells and given as the average counts per minute (cpm) of all donors + the standard error of the mean (SEM).

Generation of influenza A virus-specific CTL effector cells from PBMC

To assess influenza A virus-specific CTL activity *in vitro*, PBMC were stimulated with Resvir-9 as previously described (6). Briefly, 5 x 10^6 PBMC were infected with Resvir-9 at a MOI of 3 for 1 h at 37°C in R10F. Following centrifugation, the PBMC were resuspended in R10H and added to uninfected PBMC at a ratio of 1:1. After 2 days at 37°C, rIL-2 (50 U/ml final concentration) was added, and the cells were incubated for another 6 days. Prior to the CTL assay CD4+ cells were depleted using M-450 DYNABEADS (Dynal, Oslo, Norway), followed by the depletion of NK cells with anti-human CD16 MAb (Pharmingen) coupled to Pan anti-mouse-IgG

beads (Dynal) according to manufacturer's instructions. The remaining effector cell population was used to assess the lytic ability of effector cells. The depletion of CD4+ and CD16+ cells was validated on every occasion and the percentage CD4+ CD3+ cells and CD16+ CD3- cells was determined by flow cytometry. Contamination with these cell populations never exceeded 2.0%. The percentage CD8+ CD3+ T cells in the remaining effector cell population was determined in order to calculate the CD8:Target (CD8:T) cell ratio used in the cytotoxicity assay.

Cytotoxicity assay

Autologous BLCL were used as target cells and infected with Resvir-9 at a MOI of 1 for 1 h at 37°C, spun down, and resuspended in R10F. After 16 h at 37°C, influenza A virus-infected BLCL and uninfected BLCL were washed in serum free medium and labeled for 1 h at 37°C with 50 μCi $Na_2[^{51}Cr]O_4$ per 5 x 10^5 cells. Following three washing steps with R10F, the target cells were added to effector cells at different CD8:Target cell ratios for 4 h at 37°C and the supernatant was subsequently harvested. The percentage specific lysis was calculated with the following formula: ((experimental release – spontaneous release) / (maximal release – spontaneous release)) x 100. The average percentage specific lysis was calculated from a minimum of three wells and given as the percentage lysis of virus-infected autologous BLCL minus the percentage lysis of uninfected autologous BLCL. The slope of each plot was calculated with the logarithmic trendline between CD8:Target cell ratio and the percentage specific lysis.

Statistical analysis

The relationship between percentage cytokine positive cells and age was calculated with a two-tailed Pearson bivariate correlation assay. P values below 0.05 were considered statistically significant. The statistical significance in lytic ability of effector cells and proliferation of PBMC obtained from 18- to 20-year-old and 68- to 70-year-old donors was determined with Student's t test.

Results

Serology

The sera of all 87 donors were tested for the presence of influenza A virus-specific antibodies. Only one donor had no hemagglutination inhibition (HAI) titer against any of the influenza viruses. The remaining 86 donors had serum antibodies against influenza A virus (H1N1 or H3N2), indicating that they had been infected with influenza A virus.

Precursor frequency of influenza A virus-specific T cells

Of the 82 donors tested for the presence of influenza A virus-specific CD3+ CD8+ cells, 45 donors (55 %) were positive and had a higher percentage IFN-γ+ cells within the CD3+ CD8+ fraction of the PBMC, than the cut-off value of 0.11% (dotted line, figure 1c). Comparing the percentage of influenza A virus-specific IFN-γ+ cells in the CD3+ CD8+ population among donors of various ages, no age-related

differences were observed (average = 0.23 %, solid line, figure 1c, $P = 0.778$). In addition to CTLp frequency, the percentage of virus-specific cells in the CD3+ CD4+ population was determined and found to be lower than the frequency of virus-specific CD3+ CD8+ cells. In 33 (40%) out of 85 donors tested for virus-specific IFN-γ+ CD3+ CD4+ cells the percentage IFN-γ+ cells in the CD3+ CD4+ cells was higher than the cut-off value (dotted line, figure 1d). The average percentage IFN-γ+ cells in CD3+ CD4+ was 0.16% in these 33 donors, while the overall average percentage of virus-specific cells in the CD3+ CD4+ population was 0.07%. Again, no age-related differences were found in the percentage of IFN-γ+ CD3+ CD4+ cells (solid line, figure 1d, $P = 0.657$). Finally, the frequency of all influenza A virus-specific CD3+ cells was determined in 79 donors, of whom 56 donors (71 %) proved to be positive. The average percentage IFN-γ+ cells in the CD3+ population in all donors was 0.21%, ranging from 0.0% to 1.49%. The frequency of virus-specific cells in the CD3+ population also did not differ significantly ($P = 0.628$) between donors of different ages (data not shown).

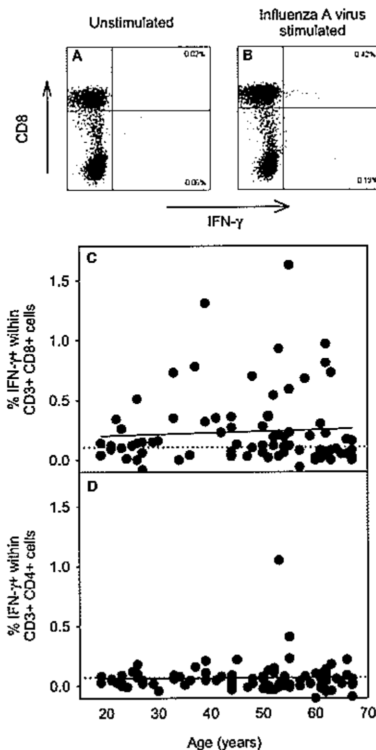

Figure 1: Precursor frequency of influenza A virus-specific T cells in fresh PBMC of donors of various ages.
PBMC were stimulated *ex vivo* with influenza A virus (H3N2, Resvir-9) as described in Materials and Methods. Figure A and B show intracellular IFN-γ staining in unstimulated (A) and influenza A virus (Resvir-9) stimulated (B) CD3+ cells. The percentage IFN-γ+ cells in a large number of donors of various ages is depicted within the CD3+ CD8+ cells (C, n = 82) and CD3+ CD4+ cells (D, n = 85). The cut-off value is represented by the dotted line (---) and the correlation between percentage IFN-γ+ cells and age by the solid line (—).

Percentage of IFN-γ and interleukin-4 producing T cells after PMA (phorbol 12-myristate 13-acetate) and ionomycin stimulation

The percentage IFN-γ+ cells within the CD3+ CD8+ population was found to increase during aging after stimulation of PBMC with PMA/ionomycin ($P < 0.001$, closed symbol, figure 2a). A similar increase, although less pronounced, was found

in CD3+ cells (P = 0.001, open symbol, figure 2a). The difference between percentage IFN-γ+ cells in the CD3+ CD8+ population and in the CD3+ population increased gradually during aging. Also the percentage IL-4 producing cells within the CD3+ CD8+ compartment increased during aging (P = 0.003, figure 2b). Since both the percentage IFN-γ+ and IL-4+ cells were determined in the CD8+ T cell fraction, the ratio between IFN-γ+ and IL-4+ CD8+ T cells was calculated in cytokine positive donors. The ratio of IFN-γ versus IL-4 producing CD3+ CD8+ cells after PMA/ionomycin stimulation increased during aging, although this change was not statistically significant (P = 0.124, figure 2c).

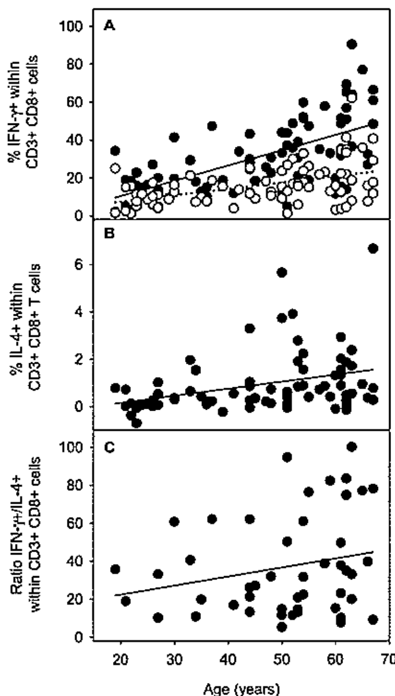

Figure 2: Percentage IFN-γ and IL-4 positive T cells in PBMC after stimulation with PMA/ionomycin.
PBMC were stimulated *ex vivo* with PMA/ionomycin for six hours at 37°C, followed by intracellular IFN-γ and IL-4 staining, as described in material and methods. (A) Percentage IFN-γ+ cells within the CD3+ CD8+ cell population (●) and CD3+ cell population (○) in donors of various ages. A significant increase in the percentage IFN-γ+ cells in the CD3+ CD8+ cells (——, $P < 0.001$) and in the CD3+ cells (----, $P = 0.001$) was observed during aging. (B) Percentage of CD3+ CD8+ cells producing IL-4 in donors of various ages. A significant increase (——, $P = 0.003$) in the percentage IL-4+ CD3+ CD8+ cells during aging was observed. (C) Ratio between IFN-γ and IL-4 producing CD3+ CD8+ cells did not change during aging (——, $P = 0.124$).

Influenza virus-specific CTL effector function in 18- to 20- and 68- to 70-year-old donors

Once it was concluded that the frequency of influenza A virus-specific CTL did not fluctuate during aging and therefore as such did not contribute to a reduced CTL activity of PBMC from the elderly it was decided to investigate the CTL activity of PBMC in greater detail. Therefore PBMC of five donors between 18 and 20 years and five donors between 68 and 70 years of age, were stimulated *in vitro* with influenza A virus.

Lymphoproliferative response of PBMC

First the PBMC obtained from the five 18- to 20- and 68- to 70-year-old donors were evaluated for signs of immunosenescence. The T cell proliferative response

after stimulation with influenza antigen at different concentrations was significantly higher in 18- to 20-year old donors than in donors 68- to 70-years of age (avg. $P = 0.025$, figure 3a). This difference was not dependent on the dose of antigen used for stimulation of the PBMC.

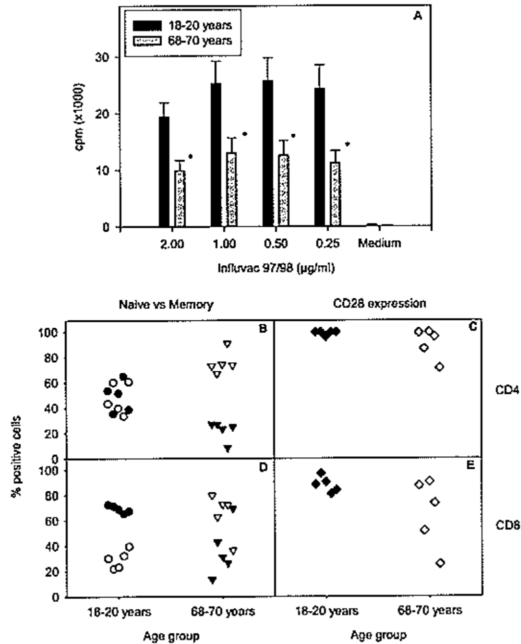

Figure 3: Characteristics of PBMC obtained from five 18-20 year old and five 68-70 year old donors.
(A) ^{3}H-thymidine incorporation after stimulation of 1 x 10^5 PBMC with a two-fold serial dilution of Influvac 97/98 (µg HA per strain/ml), including a negative control (medium). The mean counts per minute (cpm) of five donors is given and shown to differ significantly (*, $P < 0.05$) between 18-20 and 68-70 year old donors at different antigen concentrations. The data are representative of two independently repeated experiments. (B and D) CD45RA (naïve T cells, black symbols) and CD45RO (memory T cells, white symbols) expression on CD3+ CD4+ cells (B) and CD3+ CD8+ cells (D) in 18-20 year old donors (● , ○) and 68-70 year old donors (▼ , ▽). (C and E) CD28 expression on CD3+ CD4+ cells (C) and CD3+ CD8+ cells (E) in 18-20 year old donors (◆) and 68-70 year old donors (◇).

Phenotype of CD4+ and CD8+ T cells

The CD45RA, CD45RO and CD28 expression was determined in both CD4+ and CD8+ T cells. In the CD3+ CD4+ cells of 18- to 20-year-old donors the CD45RA and CD45RO expression was evenly distributed (figure 3b), while the majority of the CD3+ CD8+ cells expressed CD45RA (figure 3d). In the 68- to 70-year-old donors both the CD4+ and CD8+ T cells mainly expressed CD45RO (figure 3b and 3d). The number of CD4+ and CD8+ T cells expressing CD28 was reduced in donors 68-70 years of age, but this reduction was more pronounced in CD8+ T cells (figure 3c and 3e).

CTL activity of influenza virus-stimulated PBMC

Following *in vitro* stimulation of PBMC with influenza A virus, the CTL effector cells were tested for their ability to lyse influenza A virus-infected autologous target

cells. In figure 4, the specific lysis was plotted against the CD8:Target cell ratio for the 18- to 20-year-old (figure 4a) and the 68- to 70-year-old (figure 4b) donors, enabling us to determine the percentage specific lysis at a given CD8:Target cell ratio, which is a measure for both the lytic activity of the effector cells and the number of virus-specific cells present in the effector cell population. The average percentage specific lysis at a CD8:Target cell ratio of 10:1 was similar between both groups. The slopes of the curves are an indication for the lytic capacity of the virus-specific effector cells. The lytic capacity of the effector cells, calculated as the average correlation coefficient, seemed lower in the group of 68- to 70-year-old donors, although the difference was not statistically significant ($P = 0.25$, figure 4c).

Discussion

In the present article it was shown that precursor frequencies of influenza A virus-specific T cells remain stable during aging, indicating that the reduced CTL activity of T cells after stimulation *in vitro* in the elderly is not the result of a reduction in the numbers of virus-specific cells during aging, but more likely the result of impaired antigen specific proliferation or lower lytic capacity of these cells.
The frequency of influenza virus-specific T cells was determined *ex vivo* in fresh PBMC of donors of various ages. First, PBMC were infected with influenza A virus for 6 h, allowing the antigen presenting cells (APC) to present viral antigens in both HLA class I and II molecules on the surface of the cell (22). In the PBMC population the APC are B cells and monocytes, of which the latter can be efficiently infected by influenza virus (31). Previous studies have shown that the APC within the influenza virus-infected PBMC can stimulate high numbers (2-4%) of virus-specific CD8+ T cells (6). The induction of apoptosis through the Fas-FasL pathway, as recently described (31), does not interfere with our assay, since *in vitro* experiments with FasL-specific monoclonal antibodies (MAb) confirmed that apoptosis of T cells is a random process (unpublished observation).
The average frequency of influenza A virus-specific IFN-γ producing cells within the CD8+ T cell population remained stable during aging and was similar to previously reported precursor frequencies of EBV-, influenza A virus-, or measles virus-specific CD8+ T cells (6, 11, 30). The average percentage virus-specific CD8+ T cells (0.23%) is higher than the average percentage virus-specific CD4+ T cells. A difference in the regulation of T cell memory between CD4+ T cells and CD8+ T cells could be at the basis of a lower influenza virus-specific CD4+ T cell frequency (20). In our cohort a decline in the percentage CD3+ cells in PBMC during aging was observed ($P = 0.022$), which could conceal a possible decline in the frequency of influenza A virus-specific CTL in PBMC. However, when the frequency of virus-specific CTL was analyzed as a frequency of virus-specific CD8+ cells of all PBMC, still no significant difference ($P = 0.841$) in frequency of influenza A virus-specific cells was observed during aging. The finding that numbers of influenza A virus-specific T cells do not change during aging, even after adjusting for number of CD3+ T cells in the PBMC population, conflicts with other studies which describe a reduced IFN-γ production after stimulation with influenza virus (5, 10, 25, 32). In

these studies ELISA systems were used to measure cytokine production in culture supernatant of PBMC which had been stimulated *in vitro* for several days. Results obtained after prolonged *in vitro* stimulation do not account for differences in proliferation of lymphocytes between young and old donors (this article, (5, 18)). Using flow cytometry, cytokines can be detected in activated T cells after a short period of stimulation which makes it possible to quantify numbers of virus-specific cells without the need of *in vitro* proliferation of these cells. It could be anticipated

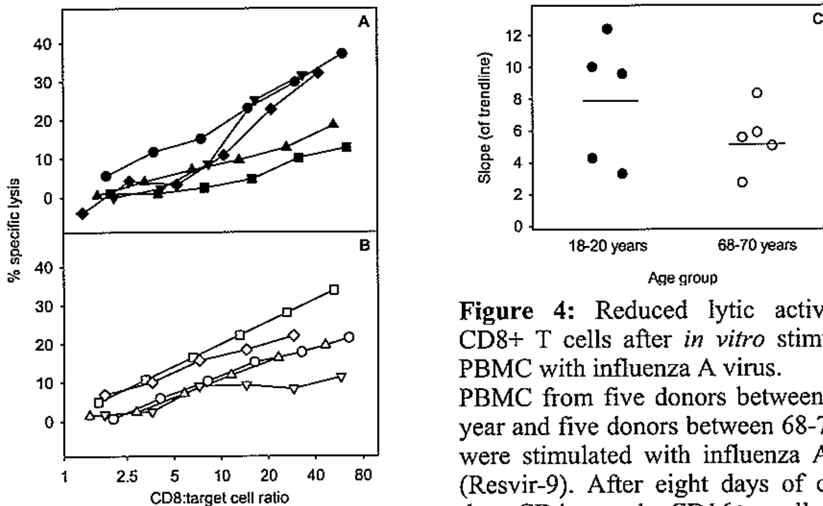

Figure 4: Reduced lytic activity of CD8+ T cells after *in vitro* stimulation PBMC with influenza A virus.
PBMC from five donors between 18-20 year and five donors between 68-70 year were stimulated with influenza A virus (Resvir-9). After eight days of culture, the CD4+ and CD16+ cells were depleted and the remaining effector cells tested for lytic capacity in a CTL assay against influenza A virus-infected or uninfected autologous target cells (BLCL) at the indicated CD8:Target cell ratio. The figure shows the percentage lysis of infected target cells minus uninfected target cells for five 18-20 year old donors (A) and five 68-70 year old donors (B). The slopes of the trendlines (specific lysis vs CD8:Target cell ratio) for the five donors between 18 and 20 years of age (●) and five donors between 68 and 70 years of age (○) was plotted (C).

that in the light of recurrent infections with influenza A virus throughout a lifetime the CTLp frequency would increase during aging. Our studies indicate however, that this is not the case and that CTLp frequencies remain stable. This would imply that after recurrent infections the number of memory CD8+ T cells return to a certain basic level.

When the IFN-γ production of PBMC upon a mitogenic stimulation between young and older individuals was compared, conflicting results have been obtained (8, 9, 14, 21, 39, 45). Our results, using flow cytometry, show a significant increase in the percentage IFN-γ+ CD8+ T cells and IFN-γ+ CD3+ cells, which is in accordance with previous studies (1, 39). The increase in the number of IFN-γ producing T cells coincides with an increased CTL memory pool in the elderly (this paper, (13, 43)). It

has been reported that after stimulation of memory T cells a large variety of cytokines, including IL-4 and IFN-γ, are produced (3). This could account for the increase in percentage IL-4+ and IFN-γ+ CD8+ T cells after PMA/ionomycin stimulation. When the frequencies of IFN-γ producing cells after stimulation with PMA and influenza A virus were compared, a significant correlation was found ($P = 0.028$). To rule out a possible effect of high PMA responders on the frequency of IFN-γ producing influenza A virus-specific CD8+ T cells, statistical analysis was performed excluding these high PMA responders (> 60% IFN-γ+ cells within CD8+ T cells). Again no statistical difference in the frequency of influenza virus-specific IFN-γ+ CD8+ T cells was found during aging ($P = 0.617$), indicating that the high PMA responders in the elderly did not mask a possible decline in the frequency of influenza virus-specific CTL during aging.
In addition to an increase in the number of IFN-γ producing CD8+ T cells also the percentage IL-4+ CD8+ T cells, after PMA/ionomycin stimulation, was increased during aging. This in contrast to a previous study (1), reporting no difference in the number of IL-4+ producing CD8+ T cells during aging. However with ELISA systems an increase in IL-4 production in CD8+ T cells of older donors was found (45). The ratio IFN-γ to IL-4 producing CD8+ T cells was increased in older donors, suggesting a shift towards Th1 phenotype of CD8+ T cells. In some studies a shift towards a Th2 response during aging is described, although these studies measured cytokine production in supernatant of *in vitro* cultured PBMC as opposed to activated CD8+ T cells obtained *ex vivo*. A shift towards Th1 phenotype was also reported after PMA/ionomycin stimulation of purified CD4+ T cells (39).
To further investigate the CTL immunity to influenza A virus in the elderly, CTL activity of purified CD8+ T cells obtained from five 68- to 70-year-old individuals was investigated and compared with that of five 18- to 20-year-old individuals. Functional and phenotypic differences between the groups indicated immunosenescence; shift from naïve to memory cell phenotype, reduced CD28 expression and lower proliferative capacity (13). The lysis of virus-infected cells by virus-specific CTL is dependent on several variables, including the number of virus-specific cells in the effector cell population and the lytic capacity of these cells. A measure of the latter can be deduced from the slope of the logarithmic trendline of the plots shown in figure 4. Our results suggest that the lytic capacity per cell is lower in older donors than in young donors, which could be explained by a reduced perforin or granzyme expression (38). More studies in larger cohorts of donors are required to further investigate the lytic capacity of CTL during aging. At certain CD8:Target ratio's (10:1) essentially no difference in CTL activity was observed between PBMC obtained from 68- to 70-year-old donors and the PBMC obtained from 18- to 20-year-old individuals. The addition of exogenous IL-2 in our experiments may have compensated for the reduction in CTL activity of *in vitro* expanded CTL observed without the addition of IL-2 (25, 26, 35). The reduced CTL activity in the elderly observed without the addition of IL-2 to PBMC cultures may indicate that the proliferation of virus-specific CD8+ T cells is reduced by defects in CD4+ T cells. Indeed, we and others have shown that the proliferation of virus-specific CD4+ T cells is severely impaired in the elderly.

Collectively, we conclude that during aging the precursor frequency of influenza A virus-specific CTL does not fluctuate. Apparently the pool of memory CTL is maintained and repeated exposure to the virus does not increase this pool. The reported reduction of CTL activity in the elderly is most likely caused by other defects in CD8+ T cells or other cells of the immune system.

References

1. **Bandres, E., J. Merino, B. Vazquez, S. Inoges, C. Moreno, M. L. Subira, and A. Sanchez-Ibarrola.** 2000. The increase of IFN-gamma production through aging correlates with the expanded CD8(+high)CD28(-)CD57(+) subpopulation. Clin. Immunol. **96:**230-235.
2. **Barker, W. H., and J. P. Mullooly.** 1980. Impact of epidemic type A influenza in a defined adult population. Am. J. Epidemiol. **112:**798-811.
3. **Beckman, I., K. Shepherd, K. Dimopoulos, M. Ahern, F. Firgaira, and J. Bradley.** 1994. Differential expression and regulation of cytokine mRNAs in normal human CD45R T cell subsets. Cytokine. **6:**116-123.
4. **Bender, B. S., T. Croghan, L. Zhang, and P. A. Small.** 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. **175:**1143-1145.
5. **Bernstein, E. D., E. M. Gardner, E. Abrutyn, P. Gross, and D. M. Murasko.** 1998. Cytokine production after influenza vaccination in a healthy elderly population. Vaccine. **16:**1722-1731.
6. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. The magnitude and specificity of influenza A virus-specific cytotoxic T lymphocyte responses in humans is related to HLA-A and -B phenotype. J. Virol. **76:**582-90.
7. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. J. Virol. **76:**2567-72.
8. **Born, J., D. Uthgenannt, C. Dodt, D. Nunninghoff, E. Ringvolt, T. Wagner, and H. L. Fehm.** 1995. Cytokine production and lymphocyte subpopulations in aged humans. An assessment during nocturnal sleep. Mech. Ageing Dev. **84:**113-126.
9. **Cakman, I., J. Rohwer, R. M. Schutz, H. Kirchner, and L. Rink.** 1996. Dysregulation between TH1 and TH2 T cell subpopulations in the elderly. Mech. Ageing Dev. **87:**197-209.
10. **Castle, S. C.** 2000. Clinical relevance of age-related immune dysfunction. Clin. Infect. Dis. **31:**578-585.
11. **Crucian, B. E., R. P. Stowe, D. L. Pierson, and C. F. Sams.** 2001. Routine detection of Epstein-Barr virus specific T cells in the peripheral blood by flow cytometry. J. Immunol. Methods. **247:**35-47.

12. **de Jong, J. C., W. E. Beyer, A. M. Palache, G. F. Rimmelzwaan, and A. D. Osterhaus.** 2000. Mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly. J. Med. Virol. **61:**94-99.
13. **Effros, R. B.** 2001. Immune system in the elderly, p. 324-350. *In* E. J. Masoro, and S. Austad (eds), Handbook of the Biology of Aging (5th edn). Academic Press, London.
14. **Fagiolo, U., A. Cossarizza, E. Scala, E. Fanales-Belasio, C. Ortolani, E. Cozzi, D. Monti, C. Franceschi, and R. Paganelli.** 1993. Increased cytokine production in mononuclear cells of healthy elderly people. Eur. J. Immunol. **23:**2375-2378.
15. **Falsey, A. R., C. K. Cunningham, W. H. Barker, R. W. Kouides, J. B. Yuen, M. Menegus, L. B. Weiner, C. A. Bonville, and R. F. Betts.** 1995. Respiratory syncytial virus and influenza A infections in the hospitalized elderly. J. Infect. Dis. **172:**389-394.
16. **Falsey, A. R., and E. E. Walsh.** 2000. Respiratory syncytial virus infection in adults. Clin. Microbiol. Rev. **13:**371-384.
17. **Fu, T. M., L. Guan, A. Friedman, T. L. Schofield, J. B. Ulmer, M. A. Liu, and J. J. Donnelly.** 1999. Dose dependence of CTL precursor frequency induced by a DNA vaccine and correlation with protective immunity against influenza virus challenge. J. Immunol. **162:**4163-4170.
18. **Globerson, A., and R. B. Effros.** 2000. Ageing of lymphocytes and lymphocytes in the aged. Immunol. Today. **21:**515-521.
19. **Gross, P. A., M. Rodstein, J. R. LaMontagne, R. A. Kaslow, A. J. Saah, S. Wallenstein, R. Neufeld, C. Denning, P. Gaerlan, and G. V. Quinnan.** 1988. Epidemiology of acute respiratory illness during an influenza outbreak in a nursing home. A prospective study. Arch. Intern. Med. **148:**559-561.
20. **Homann, D., L. Teyton, and M. B. Oldstone.** 2001. Differential regulation of antiviral T cell immunity results in stable CD8+ but declining CD4+ T cell memory. Nat. Med. **7:**913-919.
21. **Karanfilov, C. I., B. Liu, C. C. Fox, R. R. Lakshmanan, and R. L. Whisler.** 1999. Age-related defects in Th1 and Th2 cytokine production by human T cells can be dissociated from altered frequencies of CD45RA+ and CD45RO+ T cell subsets. Mech. Ageing Dev. **109:**97-112.
22. **Kuijpers, K. C., F. J. van Kemenade, B. Hooibrink, J. J. Neefjes, C. J. Lucas, R. A. van Lier, and F. Miedema.** 1992. HLA class I and II molecules present influenza virus antigens with different kinetics. Eur. J. Immunol. **22:**2339-2345.
23. **Kuwano, K., M. Tamura, and F. A. Ennis.** 1990. Cross-reactive protection against influenza A virus infections by an NS1-specific CTL clone. Virology. **178:**174-9.
24. **Masurel, N., P. Ophof, and P. de Jong.** 1981. Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. J. Hyg. (Lond). **87:**201-209.
25. **Mbawuike, I. N., C. L. Acuna, K. C. Walz, R. L. Atmar, S. B. Greenberg, and R. B. Couch.** 1997. Cytokines and impaired CD8+ CTL activity

among elderly persons and the enhancing effect of IL-12. Mech. Ageing Dev. **94**:25-39.

26. **Mbawuike, I. N., A. R. Lange, and R. B. Couch.** 1993. Diminished influenza A virus-specific MHC class I-restricted cytotoxic T lymphocyte activity among elderly persons. Viral Immunol. **6**:55-64.

27. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare.** 1983. Cytotoxic T cell immunity to influenza. N. Engl. J. Med. **309**:13-17.

28. **Miller, R. A.** 1996. The aging immune system: primer and prospectus. Science. **273**:70-74.

29. **Mlinaric-Galinovic, G., A. R. Falsey, and E. E. Walsh.** 1996. Respiratory syncytial virus infection in the elderly. Eur. J. Clin. Microbiol. Infect. Dis. **15**:777-781.

30. **Nanan, R., A. Rauch, E. Kampgen, S. Niewiesk, and H. W. Kreth.** 2000. A novel sensitive approach for frequency analysis of measles virus-specific memory T lymphocytes in healthy adults with a childhood history of natural measles. J. Gen. Virol. **81**:1313-1319.

31. **Nichols, J. E., J. A. Niles, and N. J. Roberts, Jr.** 2001. Human lymphocyte apoptosis after exposure to influenza A virus. J. Virol. **75**:5921-5929.

32. **Ouyang, Q., G. Cicek, R. G. Westendorp, H. J. Cools, R. J. van der Klis, and E. J. Remarque.** 2000. Reduced IFN-gamma production in elderly people following in vitro stimulation with influenza vaccine and endotoxin. Mech. Ageing Dev. **121**:131-137.

33. **Palmer, D., W. Dowdle, M. Coleman, and G. Schild** 1975. Haemagglutination inhibition test, p. 25-62. *In* Anonymous (ed.), Advanced laboratory techniques for influenza diagnosis. Procedural uide. US Dept. Hlth. ED., Atlanta.

34. **Pawelec, G., M. Adibzadeh, R. Solana, and I. Beckman.** 1997. The T cell in the ageing individual. Mech. Ageing Dev. **93**:35-45.

35. **Powers, D. C.** 1993. Influenza A virus-specific cytotoxic T lymphocyte activity declines with advancing age. J. Am. Geriatr. Soc. **41**:1-5.

36. **Rimmelzwaan, G. F., M. Baars, E. C. Claas, and A. D. Osterhaus.** 1998. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J. Virol. Methods. **74**:57-66.

37. **Rimmelzwaan, G. F., N. Nieuwkoop, A. Brandenburg, G. Sutter, W. E. Beyer, D. Maher, J. Bates, and A. D. Osterhaus.** 2000. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine. **19**:1180-1187.

38. **Rukavina, D., G. Laskarin, G. Rubesa, N. Strbo, I. Bedenicki, D. Manestar, M. Glavas, S. E. Christmas, and E. R. Podack.** 1998. Age-related decline of perforin expression in human cytotoxic T lymphocytes and natural killer cells. Blood. **92**:2410-2420.

39. **Sakata-Kaneko, S., Y. Wakatsuki, Y. Matsunaga, T. Usui, and T. Kita.** 2000. Altered Th1/Th2 commitment in human CD4+ T cells with ageing. Clin. Exp. Immunol. **120**:267-273.

40. **Ulmer, J. B., T. M. Fu, R. R. Deck, A. Friedman, L. Guan, C. DeWitt, X. Liu, S. Wang, M. A. Liu, J. J. Donnelly, and M. J. Caulfield.** 1998. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J. Virol. **72:**5648-5653.
41. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J. Virol. **74:**6800-6807.
42. **Wick, G., and B. Grubeck-Loebenstein.** 1997. The aging immune system: primary and secondary alterations of immune reactivity in the elderly. Exp. Gerontol. **32:**401-413.
43. **Wick, G., and B. Grubeck-Loebenstein.** 1997. Primary and secondary alterations of immune reactivity in the elderly: impact of dietary factors and disease. Immunol. Rev. **160:**171-184.
44. **Yap, K. L., G. L. Ada, and I. F. McKenzie.** 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. Nature. **273:**238-239.
45. **Yen, C. J., S. L. Lin, K. T. Huang, and R. H. Lin.** 2000. Age-associated changes in interferon-gamma and interleukin-4 secretion by purified human CD4+ and CD8+ T cells. J. Biomed. Sci. **7:**317-321.

Chapter 7

In vitro effect of bioactive compounds on influenza virus-specific B and T cell responses

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Scandinavian Journal of Immunology 2002;55:24-32

Abstract

In vitro studies have demonstrated positive effects of bioactive compounds on several functions of the immune system. In the present study, 25 of such compounds were tested for their immune modulating properties on influenza virus-specific human B and T cell responses *in vitro*. One of these compounds, N-acetyl-L-cysteine was shown to increase influenza virus-specific lymphocyte proliferation and gamma-interferon (IFN-γ) production at a concentration of 1.0 mmol/l. Furthermore, N-acetyl-L-cysteine was found to enhance specific activity of two influenza A virus-specific $CD8^+$ cytotoxic T lymphocyte clones directed towards HLA-A*0201- and HLA-B*2705-restricted epitopes. A second compound, chlorogenic acid, was shown to enhance antigen-specific proliferation of lymphocytes in three out of four donors, at concentrations of 10-50 μmol/l. Neither of the two compounds exhibited a positive effect on the production of influenza virus-specific antibodies by human peripheral blood mononuclear cells *in vitro*.

Introduction

In the last decades, interest in beneficial effects of bioactive compounds on several aspects of health has increased (5, 15, 24, 29). For example, epidemiological surveys revealed that the consumption of n-3 polyunsaturated fatty acids or soybean

Table 1: Bioactive compounds used in this study

Name of compound	Cat. no. (Source)	Stock concentration	Solvent [b]
Vitamin A_1 acid	R-7632 (Sigma)	50.0 mmol/l	EtOH [c]
Retinol acetate	R-7882 (Sigma)	60.0 mmol/l	H_2O
Vitamin B6	P-6280 (Sigma)	100 mmol/l	H_2O
Ascorbic acid	A-4544 (Sigma)	100 mmol/l	H_2O
α-Tocopherol	T-3251 (Sigma)	100 mmol/l	EtOH
Biotin	TT687714 (Merck, Darmstadt, Germany)	16.4 mmol/l	H_2O
Coenzyme Q10	C-9538 (Sigma)	1.15 mmol/l	EtOH
Arginine	A-5006 (Sigma)	100 mmol/l	H_2O
ß-carotene	ICN101287 (ICN Biomedicals Inc.)	100 μmol/l	EtOH/Hex
Astaxanthin	A-9335 (Sigma)	100 μmol/l	EtOH/Hex
Grape seed skin extract	Activin GSE-2000 (InterHealth Nutraceuticals, Benicia, CA, USA)	5.0 mg/ml	KOH
Soylife 150 extract	Extract containing 12.8% isoflavinoids (Soylife Nederland BV ,Giessen, Netherlands)	5.0 mg/ml	KOH
Taurine	T-7146 (Sigma)	500 mmol/l	H_2O
Lipoic acid	T-8260 (Sigma)	200 mmol/l	EtOH
Acetyl-L-carnitine	A-6706 (Sigma)	1.0 mol/l	H_2O
N-acetyl-L-cysteine	A-8199 (Sigma)	1.0 mol/l	H_2O
Protocatechuic acid	P-5630 (Sigma)	10.0 mmol/l	H_2O
Ferulic acid	F-3500 (Sigma)	10.0 mmol/l	H_2O
Chlorogenic acid	C-3878 (Sigma)	10.0 mmol/l	H_2O
Quercetin di-hydrate	P-102 (ICC, Belle Mead, NJ, USA)	5.0 mmol/l	KOH
Coumarin	C-4261 (Sigma)	5.0 mmol/l	KOH
Catechin	C-1251 (Sigma)	10.0 mmol/l	KOH
Daidzein	D-7802 (Sigma)	10.0 mmol/l	KOH
Genistin	G-0897 (Sigma)	10.0 mmol/l	KOH
Genistein	G-6776 (Sigma)	10.0 mmol/l	KOH

Detailed information, concerning ordering information, solubility and solvent, of the 25 dietary compounds studied for immune modulatory properties.
[b] KOH was used at a concentration of 50 mmol/l.
[c] abbreviations: KOH, potassium hydroxide: EtOH, ethanol: EtOH/Hex, ethanol/hexane (49:1)

containing diets correlated with a lower incidence of coronary heart disease and diabetes mellitus or cancer, respectively (1, 10, 31). Especially the effect on the immune system, which plays a crucial role in the defence against invading foreign microorganisms and in killing of cancer cells, has been studied extensively. In addition, the decrease of immune responsiveness in the elderly has contributed to the research in this field (12, 25, 36), since a decrease in immune responsiveness can partly be attributed to protein-energy malnutrition and decreased micronutrient levels (17, 18, 22).
Bioactive dietary compounds can be classified into several groups. The first group, derived from plants, can be further divided into several subgroups including; flavonoids (flavones, isoflavones, flavonols), coumarins, and cinnamic acids. These compounds which are present in fruit, beverages, and vegetables, exert their biological effect *in vivo* already at low concentrations. A second group is called micronutrients (vitamins, trace elements, thiols, amino acids and fatty acids), some of which are essential for biological processes (23, 26, 30).
In order to identify compounds with immune enhancing properties, a large number of bioactive compounds, were screened for their potential to enhance immune reactivity against influenza viruses *in vitro*. These compound were previously described to have immune modulating properties, such as increased antibody production, enhanced cell mediated immunity, macrophage mobility and human leukocyte antigen (HLA) molecule expression [2, 4, 16, 21, 32). Such compounds may be used as food supplements and display a beneficial effect on the function of the immune system in individuals with immune deficiencies. To this end, the effect of bioactive compounds on proliferation of human peripheral blood mononuclear cells (PBMC) and antibody production was studied, after *in vitro* stimulation with influenza virus, a virus causing high morbidity and mortality among the elderly. Out of the 25 compounds studied, two were identified which increased influenza virus-specific T cell proliferation *in vitro*: chlorogenic acid, a plant phenol with antioxidant properties, and N-acetyl-L-cysteine (NAC), a thiol antioxidant precursor of glutathion. Furthermore, the effector function of two influenza virus-specific cytotoxic T lymphocyte (CTL) clones and the interferon-γ (IFN-γ) production following stimulation with influenza antigen was increased in the presence of NAC.

Material and methods

Compounds

A total number of 25 compounds were tested for their immune modulating properties *in vitro*. The majority of these compounds belong to the families of vitamins, antioxidants, amino acids, flavonoids, plant phenols, crude extracts and coumarins (for details on solubility, solvent and source, see Tab. 1). ß-carotene and astaxanthin were dissolved, as described previously, into an ethanol:hexane mixture (49:1) and filtered with a 0.45 μm filter (OPTEX™, Millipore, Molsheim, France) (16). The final concentration was approximately 10^{-4} mol/l.

Isolation of human peripheral blood mononuclear cells

PBMC were isolated from buffy-coats obtained from nine healthy blood donors of the Bloodbank Rotterdam (Rotterdam, Netherlands). To isolate the PBMC, the blood cells were diluted 1:1 with PBS containing 10.0 mmol/l glucose and 2.5 mmol/l EDTA (washing buffer) and layered onto Lymphoprep (density: 1.077 +/- 0.001 g/ml, Nycomed, Norway). After 30 min centrifugation at 800 x *g* the PBMC were collected and washed several times in washing buffer, to remove any remaining thrombocytes. Finally the cells were washed one last time in PBS, and stored at –135°C in RPMI 1640 (Life Technologies, Rockville, USA) containing 100 IU/ml penicillin and streptomycin (Life Technologies, Rockville, USA) and 2.0 mmol/l L-glutamin (BioWhittaker Europe, Verviers, Belgium) (PSG), 20% fetal calf serum (FCS) (Greiner Labortechniek, Solingen, Germany) and 10% di-methyl sulfoxamide. Immune reactivity to influenza antigen, using a lymphocyte stimulation test (LST, see below), was established prior to further testing.

Influenza virus antigens

Influvac 97/98 (kindly provided by Solvay Pharma B.V., Weesp, Netherlands) was used to stimulate PBMC in a LST (see below) and as a coating antigen for an IgG antibody ELISA (see below). Influvac 97/98 is a trivalent vaccine composed of the glycoproteins, hemagglutinin (HA) and neuraminidase, of the influenza viruses A/Nanchang/933/95 (H3N2), A/Johannesburg/82/95 (H1N1) and B/Harbin/7/94. Before using Influvac 97/98 as an antigenic stimulus, the vaccine was dialysed overnight at 4°C against PBS to remove the preservative thiomersal. The HA concentration for each individual virus strain is 30 μg/ml. In addition, infectious and purified A/Nanchang/933/95 (Resvir-9, kindly provided by CSL Ltd., Parkville, Victoria, Australia), was used to infect PBMC.

Dose range testing

To narrow the concentration range used during assessment of immune enhancing properties of the bioactive compounds on influenza virus-specific stimulation of PBMC, each compound and the solvents were first tested for inhibitory effects on the proliferation of B-lymphoblastoid cell-lines (BLCL). BLCL from two different individuals used at a concentration of 5×10^4 cells/well in RPMI supplemented with PSG and 10% FCS (R10F) were added to a 96-well round-bottom Costar® plate (Corning Incorporated, Corning, NY, USA). The compounds were added in tenfold serial dilutions in R10F. After 24 h incubation at 37°C, 0.5 μCi/well [^{3}H]-thymidine (Amersham, Aylesbury, UK) was added and 18 h later the cells were harvested and [^{3}H]-thymidine incorporation was measured using a Beta-microplate counter (Pharmacia LKB, Turku, Finland). The assay was performed in quadruplicate and inhibition of proliferation was defined as a statistical significant reduction in proliferation of BLCL.

Lymphocyte stimulation test

The effect of bioactive compounds on the influenza virus-specific T cell response *in vitro* was measured using PBMC and purified viral glycoproteins. One hundred

thousand PBMC were stimulated with influenza virus antigens (Influvac 97/98) at a previously determined optimal concentration (1.0 μg/ml per strain) or a suboptimal

Table 2: Concentration of bioactive compounds that inhibit proliferation of BLCL and PBMC

Name of compound	Inhibitory conc. Ratio[1]	Inhibitory conc. in BLCL	Inhibitory conc. in LST
Vitamin A_1 acid [a]	1.7	0.17 mmol/l	0.1 mmol/l
Retinol acetate [a]	2	6.0 mmol/l	3.0 mmol/l
Vitamin B6	50	10.0 mmol/l	0.2 mmol/l [c]
Ascorbic acid	1	0.5 mmol/l	0.5 mmol/l
α-Tocopherol	1	> 1.0 mmol/l	> 1.0 mmol/l
Biotin	ND [d]	> 1.64 mmol/l	0.82 mmol/l [c]
Coenzyme Q10 [a]	ND	> 3.8 μmol/l	11.5 μmol/l [b]
Arginine [a]	2	10.0 mmol/l [b]	5.0 mmol/l
ß-carotene	1	1.0 μmol/l	1.0 μmol/l
Astaxanthin	ND	> 1.0 μmol/l	1.0 μmol/l [c]
Grape seed skin extract [a]	5	50.0 μg/ml	10 μg/ml
Soylife 150 extract [a]	ND	0.5 mg/ml	> 0.1 mg/ml
Taurine	ND	> 50.0 mmol/l	> 50.0 mmol/l
Lipoic acid [a]	10	10.0 mmol/l	200 μmol/l
Acetyl-L-carnitine	10	100 mmol/l	10.0 mmol/l
N-acetyl-L-cysteine	20	100 mmol/l [b]	5.0 mmol/l
Protocatechuic acid	ND	0.1 mmol/l	> 16.7 μmol/l
Ferulic acid	1	0.1 mmol/l [b]	0.1 mmol/l
Chlorogenic acid	1	0.5 mmol/l	0.5 mmol/l
Quercetin di-hydrate	10	0.5 mmol/l	0.05 mmol/l [c]
Coumarin	ND	> 0.5 mmol/l	0.25 mmol/l [c]
Catechin	10	1.0 mmol/l	0.1 mmol/l
Daidzein	10	0.5 mmol/l	0.05 mmol/l [c]
Genistin	10	1.0 mmol/l	0.1 mmol/l [c]
Genistein [a]	2	20 μmol/l	10 μmol/l [b]
EtOH	1	1:100	1:100
EtOH/hexane	1	1:100	1:100
H_2O	2	1:10	1:10 [c]
KOH (50 mmol/l)	1	1:5	1:5

Concentration of bioactive compounds with no significant inhibitory effect on proliferation of 5 x 10^4 BLCL ($P < 0.01$) and 10^5 PBMC ($P < 0.05$) stimulated with 1.0 μg/ml per strain Influvac 97/98. [1] Inhibitory concentration ratio was calculated from the concentration of a compound necessary to inhibit the proliferation of 1 x 10^4 BLCL divided by the concentration of the same compound necessary to inhibit the proliferation of 1 x 10^5 PBMC stimulated with 1.0 μg/ml per strain Influvac 97/98. [a] Bioactive compound is tested for modulatory effects on antigen specific proliferation in 2 donors, [b] Bioactive compound concentration inhibits proliferation in 1 out of 2 donors, [c] Bioactive compound concentration inhibits proliferation in 3 out of 4 donor. [d] abbreviations: KOH, potassium hydroxide; EtOH, ethanol; EtOH/Hex, ethanol/hexane; BLCL, B-lymphoblastoid cell-line; LST, lymphocyte stimulation test

concentration (0.2 µg/ml per strain) and cultured in 96-well round bottom plates in 150 µl RPMI with PSG and 10% pooled human AB serum (R10H). Non-stimulated cultures and cultures stimulated in the presence of the solvents were included as controls. To the stimulated cultures different concentrations of each of the compounds listed in table 1 and their respective solvents were added. After an optimal 5-day incubation at 37°C, 0.5 µCi/well [^{3}H]-thymidine was added followed by a subsequent 18 h incubation at 37°C. The cells were harvested and [^{3}H]-thymidine incorporation was measured with a Beta-microplate counter. A minimum of two and a maximal of four donors were used to assess immune enhancing properties of the bioactive compounds. The results were expressed as the average counts per minute (cpm) of triplicate or sextuplicate wells + SD and compared with the cpm of influenza virus antigen stimulated PBMC without the addition of bioactive compounds.

Cytokine production assay
Cytokine levels in the culture supernatants of 10^5 PBMC stimulated with Influvac 97/98 (1.0 µg/ml per strain) in the absence or presence of selected compounds were determined. Selection of the compounds for further evaluation was based on the results obtained in the LST. The stimulation protocol was similar to the previously described LST (see above), however after 5 days the supernatant was tested for the presence of interleukin-4 (IL-4) and IFN-γ, using cytokine ELISA-kits (BioSource Europe S.A., Fleurus, Belgium), according to manufacturers instructions.

In vitro antibody production assay
An *in vitro* antibody production assay was used to solely assess the effect of the respective compounds on the antibody production by B cells *in vitro*, therefore adding the compounds 5 days after the initiation of the immune reactivation. Briefly, 2 x 10^6 human PBMC were added to a 24-wells Costar® plate in duplicate in 1.0 ml R10F and infected with A/Nanchang/933/95 at a multiplicity of infection (MOI) of 0.4. R10F was used during this assay instead of R10H, because of the presence of influenza virus-specific antibodies in R10H, which would give false positive results in the antibody production assay. After 6 days at 37°C duplicate wells were pooled, washed once in R10F and redistributed in half the volume of R10F in four wells in a 96-wells flat bottom plate (CellStar®, Greiner labortechniek). The compounds were added in two of these wells, while the remaining two wells were used as medium controls. After a subsequent 2 day incubation at 37°C the cell culture supernatant was harvested, stored at –20°C and tested for the presence of influenza virus-specific IgG antibodies by ELISA (see below). Non-stimulated PBMC and the respective solvents of the compounds were included as controls in each assay.

Antibody ELISA
Flat bottom microtiter plates (Costar® EIA plates) were coated with purified influenza virus antigen (Influvac 97/98). One hundred microliter volumes containing 300 ng glycoproteins of each of the vaccine strains in 0.1 mol/l carbonate buffer (pH = 9.6) were incubated for 16 h at 4°C. Subsequently the plates were washed three

times with PBS containing 0.05% Tween-20 (PBST) and blocked with R10F for 2 h at 37°C. Supernatant (100 μl), obtained from PBMC cultures, was added to the wells and incubated for 3 h at 37°C. Influenza virus-specific IgG antibodies were detected with 100 μl of a biotin-labelled rabbit anti-human IgG antibody preparation (E0482, Dako, Glostrup, Denmark) for 1 h at 37°C, followed by an incubation with streptavidin-HRPO (Dako) for 1 h at room temperature. After every incubation the plates were washed three times with PBST. Finally, 0.1 ml substrate solution (0.1 mg/ml tetra methyl benzidine (Sigma, St. Louis, MO, USA) and 0.003% H_2O_2 in 0.1 mol/l sodium acetate buffer (pH = 5.5)) was added to each well. After 10 min incubation at room temperature, 0.1 ml 2.0 mol/l H_2SO_4 was added to stop the colour reaction and the optical density at 450 nm was measured.

Cytotoxic T cell assay

CTL-assays were performed as previously described (34). Briefly, HLA-matched BLCL were treated overnight at 37°C with 10 μmol/l of peptide in R10F and used as target cells. The two peptides (manufactured by Eurogentec (Seraing, Belgium) represent an HLA-A*0201-restricted epitope from the influenza matrix protein ($M1_{58-66}$, GILGFVFTL) (11) and an HLA-B*2705-restricted epitope derived from the influenza A nucleoprotein ($NP_{383-391}$, SRYWAIRTR) (14). The following day, the cells were washed in serum free medium, incubated for 1 h at 37°C with $Na_2[^{51}Cr]O_4$ (75 μCi/1 x 10^6 cells), gently washed 3 times in R10F and added at a final concentration of 1 x 10^4 cells/well in a V-bottom 96-wells plate (Costar®). CTL clones directed against the HLA-A*0201-restricted epitope ($M1_{58-66}$) and the HLA-B*2705-restricted epitope ($NP_{383-390}$) of influenza virus were used as effector cells at several effector to target cell ratio's (E:T-ratio). After incubation for 4 h at 37°C the supernatant of each well was harvested using a macrowell tube strip system (Skatron instruments, Sterling, Va, USA) and ^{51}Cr content was measured in a gamma counter. Compounds were added during the 4 h incubation. Preincubation of the CTL clone with 1.0 mmol/l NAC was done 2 h before the CTL-assay, and washed away before the effector cells were added to the target cells. The target cells were preincubated 1 h with 1.0 mmol/l NAC before they were labelled with $Na_2{}^{51}CrO_4$. Percentage specific ^{51}Cr-release was calculated with the following formula: (cpm experimental release – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release) x 100% and compared with untreated target cells of peptide treated target cells in the presence of 1.0 mmol/l NAC. The cpm release represents the average of 4-10 wells +/- the SD.

Intra-cellular perforin staining

The CTL clones were also used to assess the effect of NAC on the release of intra-cellular perforin upon stimulation with peptide treated BLCL. HLA-A*0201-matched BLCL were incubated overnight in R10F with 10 μmol/l peptide (GILGFVFTL). Prior to the assay peptide treated BLCL and untreated BLCL were washed twice in R10F and 1 x 10^5 cells were added to a 96-wells V-bottom plate. The clones were washed in R10F and 2 x 10^5 cells were added to the target cells in a final volume of 150 μl of R10F. After 30-120 min incubation at 37°C the cells were

pelleted by centrifugation, washed once in PBS and fixed for 20 min using a 4% paraformaldehyde (PFA) solution. All the subsequent steps were performed at 4°C. Following centrifugation, 0.2 mol/l glycine was added for 20 min and the cells were permeabilized with permeabilization buffer (PBS containing 5.0% FCS, 1.0% normal mouse serum (ICN Biomedicals Inc., Aurora, Ohio, USA) and 0.1% saponin (w/v) (Sigma) for 1 h. After centrifugation the cells were incubated for 1 h in permeabilization buffer containing FITC-conjugated anti-human perforin mouse-monoclonal antibody (MAb) (6722KK, BD Pharmingen, CA, USA) and RPE-Cy5-conjugated anti-human CD8 mouse-MAb (C7079, Dako). An FITC-conjugated IgG_{2b} mouse-MAb was used as an isotype control to exclude non-specific binding of anti-human perforin mouse MAb. The average mean-fluorescence of $CD8^+$ cells is a measure for the amount of intra-cellular perforin present at any given time point in the CTL clone, and represents the average of two individual wells.

Statistical analysis
Data were analysed using the Student's *t* test for the effect of bioactive compounds on the proliferation of BLCL and antigen stimulated PBMC, CTL activity, antibody production and cytokine production. Significance in the BLCL toxicity assay and cytokine production assay was set at $P < 0.01$, while $P < 0.05$ was considered significant in the LST, CTL assay and antibody production assay.

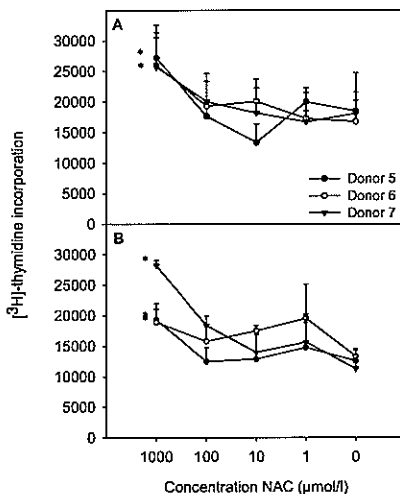


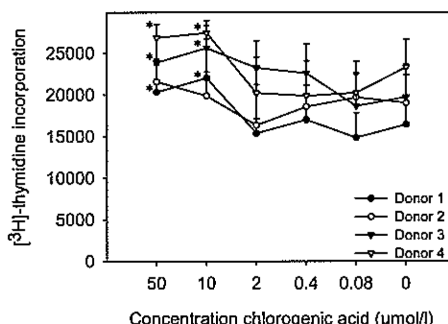
Figure 1: N-acetyl-L-cysteine (NAC) enhances influenza virus-specific proliferative T cell responses *in vitro*.
$[^3H]$-thymidine incorporation was measured 5 days after the stimulation of 10^5 PBMC with (A) 1.0 µg/ml per strain Influvac 97/98 or (B) 0.2 µg/ml per strain Influvac 97/98 in the presence of the indicated concentrations of NAC. Each data point represents the average cpm + SD of three individual wells. A significant increase in proliferation was measured in donor 5 (●) and 7 (▼) after stimulation with 1.0 µg/ml per strain of Influvac 97/98 and in all three donors after stimulation with 0.2 µg/ml per strain Influvac 97/98.
* Indicates a significant increase in proliferation ($P < 0.05$).

Results

Dose range testing

The 25 compounds and their respective solvents were tested in serial dilutions for their effect on the proliferation of BLCL, in order to identify non-inhibitory concentrations. These inhibitory concentrations, both solvents and compounds, were

Figure 2: Chlorogenic acid enhances influenza virus-specific proliferative T cell responses *in vitro*.
$[^3H]$-thymidine incorporation was measured 5 d after stimulation of 10^5 PBMC with 1.0 µg/ml per strain Influvac 97/98 in the presence of the indicated chlorogenic acid concentrations. Each data point represents the average cpm + SD of six wells. A significant increase in proliferation was observed with PBMC of donors 1 (●), 3 (▼) and 4 (▽), after the addition of 10 µmol/l or 50 µmol/l chlorogenic acid ($P < 0.05$).
* Indicates a significant increase in lymphocyte proliferation ($P < 0.05$).



excluded from any further screening of immune enhancing effects of the bioactive compounds on influenza virus stimulated PBMC. Ethanol and the ethanol:hexane mixture had no effect on proliferation of BLCL when diluted 1:100 in R10F. In addition, H_2O and KOH (50 mmol/l) could be diluted 1:10 in R10F without any effect on BLCL proliferation. The majority of the compounds, when added to the BLCL cultures at high concentrations showed an inhibition of proliferation (Tab. 2). A second group of compounds had, within the concentration range tested, no negative effect on BLCL proliferation. None of the compounds significantly increased the proliferation of BLCL. However, daidzein at a concentration of 0.1 mmol/l and α-tocopherol (2.0 mmol/l) showed an increase in BLCL proliferation from two donors in independently repeated assays. The concentration at which a particular compound with its respective solvent produced no negative effects on the proliferation of BLCL was further tested for bioactivity on influenza virus stimulated PBMC.

Effect of bioactive compounds on influenza virus-specific lymphoproliferative response in vitro

The capacity of bioactive compounds to enhance the *in vitro* lymphoproliferative response to influenza antigen was tested in the LST. The highest concentration of the compound with solvent mixture, which did not inhibit the proliferation of BLCL, was used in the LST. In addition, several 5-10 fold serial dilutions were tested. The

majority of the compounds had a negative effect at higher concentrations on the proliferation of influenza virus antigen specific PBMC (Tab. 2). When these compounds were added at lower concentrations, no effect on the influenza specific T cell response was observed, with the exception of two compounds which increased the *in vitro* influenza specific T cell response. NAC significantly increased ($P < 0.05$) the proliferation of PBMC with 40-50%, at a concentration of 1.0 mmol/l, in 2 out of 3 donors after stimulation with an optimal dose of influenza antigen (figure 1a). Following stimulation with a suboptimal dose, 1.0 mmol/l NAC significantly increased ($P < 0.05$) proliferation in all 3 donors with 40-250% (figure 1b).

In the presence of 10-50 µg/ml chlorogenic acid the influenza virus-specific lymphoproliferation was significantly increased (P < 0.05) in 3 out of 4 donors (figure 2), when compared with the control cultures. The 15-34% increase in proliferation was only observed when an optimal dose of influenza antigen was used. No increase in proliferation of unstimulated PBMC was observed in the presence of both NAC and chlorogenic acid. The results are representative for multiple assays performed independently.

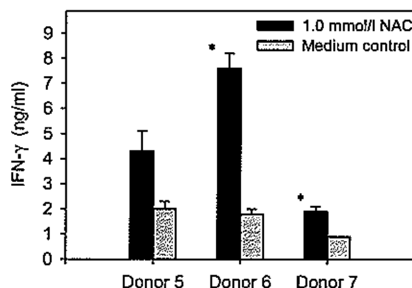


Figure 3: Increased influenza antigen induced production of interferon (IFN)-γ in the presence of N-acetyl-L-cysteine (NAC). *In vitro* IFN-γ production after 5 d by 10^5 PBMC stimulated with 1.0 µg/ml per strain Influvac 97/98 in three donors in the presence of 1.0 mmol/l NAC (black bars) compared with medium control (grey bars). Donor 6 and 7 showed significant ($P < 0.01$) increases in IFN-γ production. Each data point represents the average IFN-γ production + SD of 2 individual wells.
* Indicates a significant increase in IFN-γ production ($P < 0.01$).

Effect of NAC and chlorogenic acid on in vitro cytokine production

Compounds with immune enhancing properties, identified in the LST, were also used to study the cytokine production by PBMC after stimulation with influenza virus antigen. The addition of 1.0 mmol/l NAC increased the antigen induced IFN-γ production in all three donors in two independently performed assays (figure 3). The increase proved to be statistically significant for two out of three donors ($P < 0.01$). In unstimulated control cultures no IFN-γ production was observed in the absence or presence of 1.0 mmol/l NAC (data not shown). The increase in IFN-γ production was 2-fold in two donors and 4-fold in a third donor (donor 6). The IL-4 production remained below the detection level and was not elevated above the detection level after the addition of 1.0 mmol/l NAC (data not shown). The addition of 10-50 µmol/l chlorogenic acid did not increase the IL-4 and IFN-γ production in PBMC after an antigen specific stimulus.

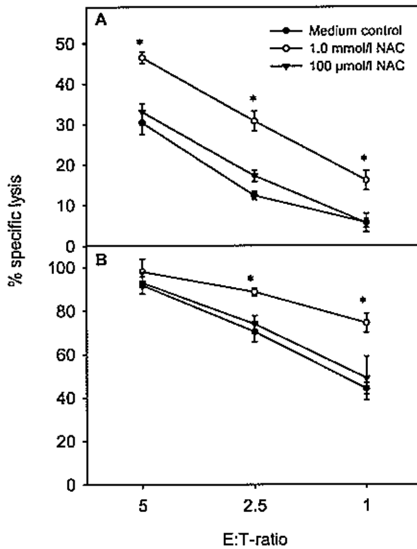


Figure 4: N-acetyl-L-cysteine (NAC) improves specific lysis of target cells by CTL-clones.
Peptide treated BLCL were used as target cells in a CTL assay and incubated with (A) a CTL clone specific for the HLA-A*0201-restricted epitope $M1_{58-66}$ of the influenza A matrix protein and (B) a CTL clone specific for the HLA-B*2705-restricted epitope $NP_{383-390}$ of the influenza A (H3N2) nucleoprotein, at the indicated effector-to-target cell (E:T) ratios. The addition of 1.0 mmol/l NAC significantly ($P < 0.01$) increased the specific lysis of target cells by both CTL clones compared with the addition of 100 µmol/l NAC or the medium control. Each value represents the average % specific lysis of four individual wells +/- the SD and the results are representative of several assays.
* Indicates a significant increase in specific lysis ($P < 0.01$).

Effect of NAC and chlorogenic acid on in vitro CTL activity
A significant increase ($P < 0.01$) in lysis during the 4 h incubation of the influenza virus-specific CTL clones with their target cells at different E:T ratios, was observed in the presence of 1.0 mmol/l NAC compared with the medium control and 100 µmol/l NAC (figure 4). This effect was predominantly attributed to CTL activity, because a two-hour preincubation of the CTL clone resulted in a pronounced increase of the specific lysis, comparable to the increase observed in CTL activity when 1.0 mmol/l NAC was added during the CTL assay (figure 5a). Furthermore, the preincubation of the target cells with 1.0 mmol/l NAC was found to have a positive effect on the specific killing of the target cells (figure 5b). The effect of the addition of 1.0 mmol/l NAC on the amount of intracellular perforin in the CTL clone was tested at several time points after stimulation with peptide treated BLCL or control BLCL. A rapid decrease in the amount of intracellular perforin in $CD8^+$ cells was observed in the presence of peptide treated BLCL, however the release of perforin was not influenced by the presence of 1.0 mmol/l NAC (figure 6). Untreated BLCL did not trigger the release of intracellular perforin contents of the CTL clone. The addition of 1.0 mmol/l NAC did not upregulate the HLA class I expression on BLCL (data not shown). Finally, no effect of 10-50 µmol/l chlorogenic acid was observed on the lytic capacity of the influenza virus-specific CTL clones (data not shown).

In vitro antibody production in the presence of bioactive compounds
The effect on antibody production *in vitro* was tested after a 5-day prestimulation of PBMC with influenza virus. The highest non-inhibitory concentration, determined in the LST, was added for a further 2 days, in which virus-specific IgG antibodies were produced. The addition of 1.0 mmol/l NAC or 50 µmol/l chlorogenic acid had no effect on the antibody production in two different donors (data not shown). Unstimulated PBMC, with or without the addition of compounds, did not produce virus-specific antibodies. The remaining compounds also failed to increase the production of influenza virus-specific antibody *in vitro* (data not shown).

Discussion

In the present paper 25 bioactive compounds were screened for their *in vitro* immune-enhancing effects. It was shown that *in vitro* NAC and chlorogenic acid increased the influenza virus antigen specific proliferation of T cells significantly. Chlorogenic acid, which is present at high concentrations in eggplant, green coffee beans and apple (19, 27), has been shown to inhibit the complement system (7) and to have no effect on other immune functions (8). It is not known whether chlorogenic acid can reach immune-enhancing concentrations *in vivo* and further investigations are required to demonstrate *in vivo* effects of chlorogenic acid.
A second bioactive compound, NAC, augmented the influenza virus-specific lymphoproliferation 1.5-2-fold in two out of three donors. The results are in agreement with those obtained by others, who reported a two-fold increase in proliferation of PBMC after a mitogenic stimulus in the presence of 5.0 mmol/l

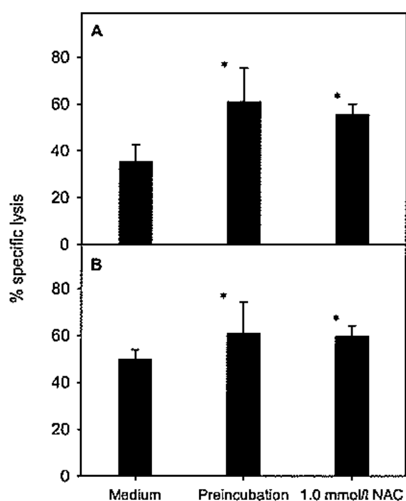


Figure 5: Preincubation of CTL and target cells with N-acetyl-L-cysteine (NAC) enhances specific lysis of target cells.
Percentage specific lysis of peptide treated target cells (BLCL) by the HLA-B*2705-restricted nucleoprotein specific CTL clone at an effector to target cell ratio of 5:1 is presented. The two graphs represent the preincubation of the CTL-clone (A) or target cell (B) with 1.0 mmol/l NAC. The bars in each individual graph correspond to the medium control (Medium), preincubation with 1.0 mmol/l NAC (preincubation) and the addition of 1.0 mmol/l NAC during the CTL assay (1.0 mmol/l NAC) as indicated. The results are the average % specific lysis measured in 20 wells + SD
* Indicates a significant increase in specific lysis ($P < 0.01$).

NAC (9). In addition, it was found that NAC increased IFN-γ production after stimulation with influenza virus, which was previously described for stimulation of PBMC with anti-CD3 (3). Furthermore, NAC was found to increase cytolytic function of antigen specific cytotoxic T cells. This effect could be attributed predominantly to the action of NAC on the CTL clone, since preincubation with 1.0 mmol/l NAC significantly increased the lytic function of CTL clone to similar levels compared with the addition of 1.0 mmol/l NAC during the 4 h incubation. However, the increased cytolytic function was not a result of a faster release of perforin within the first 2 h of the CTL-assay. A positive effect on the specific lysis, although to a lesser extent, was also observed after preincubation of the target cells with 1.0 mmol/l NAC. This effect could not be explained by an upregulation of MHC class I expression on the target cells. Another possible cause of the enhanced cytolytic function was reported by Malorni et al (20), who identified an increase in cytolytic function of natural killer (NK) cells to a NK sensitive cell-line (K562), owing to enhanced conjugate formation between the NK cell and the target cell.
The concentration at which NAC enhanced multiple immunological parameters *in vitro* (1.0 mmol/l, as described in this report) was previously reported to be obtainable *in vivo* (13). In addition, it is described that the long-term treatment with NAC reduces symptomatology and increases cell-mediated immunity after an influenza infection in elderly subjects (6). From these data it can be concluded that NAC has a favourable effect on the immune system. Immune enhancing properties were not demonstrated for the remaining 23 compounds that were tested. Addition of higher concentrations was in some cases not possible owing to toxic effects of the solvent. A large group of flavonoids has also been assessed for enhancing properties on the influenza virus-specific T cell response *in vitro*. The compounds were generally immune suppressive, with genistein being the most potent, followed by quercitin di-hydrate and daidzein, catechin, genistin. These results were in

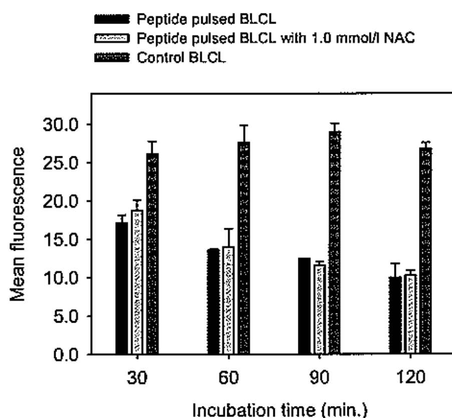


Figure 6: Perforin release in a CTL-clone is not influenced by the presence of 1.0 mmol/l N-acetyl-L-cysteine (NAC).
Average amount of intra-cellular perforin (mean fluorescence) in a CTL-clone, specific for the HLA-A*0201-restricted epitope $M1_{58-66}$ of the influenza A virus matrix protein, incubated with peptide treated BLCL in the absence of 1.0 mmol/l NAC (black bars), or the presence of 1.0 mmol/l NAC (light grey bars), and control BLCL (dark grey bars), at the indicated time points after stimulation. The CTL clone was incubated with the BLCL at an effector to target cell ratio of 2:1. The results are the average mean fluorescence + SD of two separate wells.

agreement with previous reports describing a reduced generation and effector function of CTL in mice after the addition of flavonoids (28).
The absence of immune-modulating properties of several compounds, like daidzein (35), vitamin E (33), which have been shown previously to have immune enhancing effects may have several reasons: the presence of several vitamins and amino acids in the serum and medium used for the different assays; differences in experimental design, e.g. *in vitro* versus *in vivo* studies; influenza antigen versus mitogen induced T cell proliferation; and functional differences between human and mouse lymphocytes. The concentrations, which inhibited the proliferation of BLCL and the antigen-induced proliferation of PBMC, were similar for most of the compounds. However the difference in inhibitory concentrations for the antigen specific T cell proliferation versus BLCL proliferation of some compounds like vitamin B6, lipoic acid and several flavonoids was between 10-fold and 50-fold (Tab. 2). This may indicate that these compounds have an effect on T cells or the antigen-presenting cell (APC).
In conclusion, this is the first report to describe a positive effect of chlorogenic acid on antigen-specific lymphoproliferation. The augmentation of multiple *in vitro* immunological parameters by NAC suggests a beneficial effect of NAC on immune responsiveness to invading pathogens, including influenza virus, and may lead to a reduced symptomatology (6). The immune-modulating properties of these compounds may perhaps be more potent in immune-compromised individuals, like the elderly, although this remains to be examined.

References

1. **Bang, H. O., J. Dyerberg, and H. M. Sinclair.** 1980. The composition of the Eskimo food in north western Greenland. Am. J. Clin. Nutr. **33:**2657-61.
2. **Barbieri, B., B. Lund, B. Lundstrom, and F. Scaglione.** 1999. Coenzyme Q10 administration increases antibody titer in hepatitis B vaccinated volunteers--a single blind placebo-controlled and randomized clinical study. Biofactors. **9:**351-7.
3. **Breithaupt, T. B., A. Vazquez, I. Baez, and E. H. Eylar.** 1996. The suppression of T cell function and NF(kappa)B expression by serine protease inhibitors is blocked by N-acetylcysteine. Cell. Immunol. **173:**124-30.
4. **Chew, B. P., J. S. Park, T. S. Wong, H. W. Kim, B. B. Weng, K. M. Byrne, M. G. Hayek, and G. A. Reinhart.** 2000. Dietary beta-carotene stimulates cell-mediated and humoral immune response in dogs. J. Nutr. **130:**1910-3.
5. **Craig, W. J.** 1999. Health-promoting properties of common herbs. Am. J. Clin. Nutr. **70:**491S-499S.
6. **De Flora, S., C. Grassi, and L. Carati.** 1997. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. Eur. Respir. J. **10:**1535-41.
7. **Ejzemberg, R., M. H. Da Silva, L. Pinto, and W. B. Mors.** 1999. Action of chlorogenic acid on the complement system. An. Acad. Bras. Cienc. **71:**273-7.

8. **Exon, J. H., B. A. Magnuson, E. H. South, and K. Hendrix.** 1998. Effect of dietary chlorogenic acid on multiple immune functions and formation of aberrant crypt foci in rats. J. Toxicol. Environ. Health A. **53:**375-84.
9. **Eylar, E., C. Rivera-Quinones, C. Molina, I. Baez, F. Molina, and C. M. Mercado.** 1993. N-acetylcysteine enhances T cell functions and T cell growth in culture. Int. Immunol. **5:**97-101.
10. **Feskens, E. J., and D. Kromhout.** 1993. Epidemiologic studies on Eskimos and fish intake. Ann. N. Y. Acad. Sci. **683:**9-15.
11. **Gotch, F., A. McMichael, G. Smith, and B. Moss.** 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. J. Exp. Med. **165:**408-16.
12. **Grubeck-Loebenstein, B., P. Berger, M. Saurwein-Teissl, K. Zisterer, and G. Wick.** 1998. No immunity for the elderly [letter]. Nat. Med. **4:**870.
13. **Holdiness, M. R.** 1991. Clinical pharmacokinetics of N-acetylcysteine. Clin. Pharmacokinet. **20:**123-34.
14. **Huet, S., D. F. Nixon, J. B. Rothbard, A. Townsend, S. A. Ellis, and A. J. McMichael.** 1990. Structural homologies between two HLA B27-restricted peptides suggest residues important for interaction with HLA B27. Int. Immunol. **2:**311-6.
15. **Hughes, D. A.** 1999. Effects of dietary antioxidants on the immune function of middle-aged adults. Proc. Nutr. Soc. **58:**79-84.
16. **Jyonouchi, H., S. Sun, M. Mizokami, and M. D. Gross.** 1996. Effects of various carotenoids on cloned, effector-stage T-helper cell activity. Nutr. Cancer. **26:**313-24.
17. **Lesourd, B., and L. Mazari.** 1999. Nutrition and immunity in the elderly. Proc. Nutr. Soc. **58:**685-95.
18. **Lesourd, B. M., L. Mazari, and M. Ferry.** 1998. The role of nutrition in immunity in the aged. Nutr. Rev. **56:**S113-25.
19. **Lo, H. H., and J. G. Chung.** 1999. The effects of plant phenolics, caffeic acid, chlorogenic acid and ferulic acid on arylamine N-acetyltransferase activities in human gastrointestinal microflora. Anticancer Res. **19:**133-9.
20. **Malorni, W., A. D'Ambrosio, G. Rainaldi, R. Rivabene, and M. Viora.** 1994. Thiol supplier N-acetylcysteine enhances conjugate formation between natural killer cells and K562 or U937 targets but increases the lytic function only against the latter. Immunol. Lett. **43:**209-14.
21. **Marshall, M. E., J. L. Rhoades, C. Mattingly, and C. D. Jennings.** 1991. Coumarin (1,2-benzopyrone) enhances DR and DQ antigen expressions by peripheral blood mononuclear cells in vitro. Mol Biother. **3:**204-6.
22. **Mazari, L., and B. M. Lesourd.** 1998. Nutritional influences on immune response in healthy aged persons. Mech. Ageing Dev. **104:**25-40.
23. **McKenzie, R. C., T. S. Rafferty, and G. J. Beckett.** 1998. Selenium: an essential element for immune function. Immunol. Today. **19:**342-5.
24. **Miles, E. A., and P. C. Calder.** 1998. Modulation of immune function by dietary fatty acids. Proc. Nutr. Soc. **57:**277-92.
25. **Miller, R. A.** 1996. The aging immune system: primer and prospectus. Science. **273:**70-4.

26. **Mocchegiani, E., M. Muzzioli, C. Cipriano, and R. Giacconi.** 1998. Zinc, T cell pathways, aging: role of metallothioneins. Mech. Ageing Dev. **106:**183-204.
27. **Paganga, G., N. Miller, and C. A. Rice-Evans.** 1999. The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute? Free Radic. Res. **30:**153-62.
28. **Schwartz, A., and E. Middleton, Jr.** 1984. Comparison of the effects of quercetin with those of other flavonoids on the generation and effector function of cytotoxic T lymphocytes. Immunopharmacology. **7:**115-26.
29. **Scrimshaw, N. S., and J. P. SanGiovanni.** 1997. Synergism of nutrition, infection, and immunity: an overview. Am. J. Clin. Nutr. **66:**464S-477S.
30. **Semba, R. D.** 1998. The role of vitamin A and related retinoids in immune function. Nutr. Rev. **56:**S38-48.
31. **Setchell, K. D., S. P. Borriello, P. Hulme, D. N. Kirk, and M. Axelson.** 1984. Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am. J. Clin. Nutr. **40:**569-78.
32. **Tatefuji, T., N. Izumi, T. Ohta, S. Arai, M. Ikeda, and M. Kurimoto.** 1996. Isolation and identification of compounds from Brazilian propolis which enhance macrophage spreading and mobility. Biol Pharm Bull. **19:**966-70.
33. **Tu, K. Y., R. Matthews, and K. S. Matthews.** 1995. Protective effect of vitamin E on lymphocyte growth capacity during incubation in vitro. Mech. Ageing Dev. **82:**129-48.
34. **Van Baalen, C. A., M. Schutten, R. C. Huisman, P. H. Boers, R. A. Gruters, and A. D. Osterhaus.** 1998. Kinetics of antiviral activity by human immunodeficiency virus type 1-specific cytotoxic T lymphocytes (CTL) and rapid selection of CTL escape virus in vitro. J. Virol. **72:**6851-7.
35. **Wang, W., C. M. Higuchi, and R. Zhang.** 1997. Individual and combinatory effects of soy isoflavones on the in vitro potentiation of lymphocyte activation. Nutr. Cancer. **29:**29-34.
36. **Wick, G., and B. Grubeck-Loebenstein.** 1997. The aging immune system: primary and secondary alterations of immune reactivity in the elderly. Exp. Gerontol. **32:**401-13.

Chapter 8

Summarizing Discussion

It is well known that CTL contribute to controlling virus infections, including those caused by influenza viruses. Therefore, various vaccine candidates that aim at the induction of virus-specific CTL, like live-attenuated vaccines, virosomes and immune-stimulating complexes, are evaluated at present. The studies presented in this thesis were undertaken to investigate influenza virus-specific CTL-mediated immunity and several factors that may influence the induction and functionality of CTL-mediated immunity.

Immunodominance and preferred HLA usage in the influenza virus-specific CTL response

Virus-specific CTL responses are directed against a limited number of peptides derived from viral proteins. The CTL response to certain so-called immunodominant epitopes is greater that to others, which is referred to as "immunodominance" (78, 80). Several factors contributing to immunodominance have been identified. These include the available TCR repertoire, IFN-γ expression, epitope abundance, epitope binding affinity, antigen processing and HLA expression (3, 7, 12, 19, 52, 71, 72). The effect of HLA background on immunodominance was first described in mouse models in which it was shown that the H-$2D^b$-restricted influenza A virus $PA_{224-232}$ epitope-specific CTL response was reduced in the presence of a second MHC class I allele (H-$2K^k$) (7, 24, 71). It was suggested that differences in TCR repertoire or the level of MHC class I expression were responsible for changes in the magnitude of epitope-specific CTL responses (7, 71).
In humans, the effect of HLA class I background on immunodominance profiles has not been studied in great detail. We decided to investigate the effect of HLA class I background on the influenza A virus-specific CTL response in PBMC of HLA-genotyped blood donors (chapter 2 and 3). The donors were divided into three groups of HLA-A and –B identical donors, each group sharing two or three HLA-A or –B alleles with the other groups. The HLA haplotypes of these donors were selected based on the prevalence in the Dutch population (67), the availability of HLA class I-restricted influenza A virus-specific CTL epitopes (Tab. 2, chapter 1) and the existence of HLA-transfected C1R cell-lines. The donors were between 30 and 55 years of age, in order to exclude negative effects of old age on functioning of the immune system (for review, (58)). Furthermore, the donors must have been infected at least twice with influenza A virus, which is a requirement for the imprinting of CTL memory against immunodominant epitopes (10, 13).
The immunodominance hierarchies of virus-specific CTL epitopes was determined *ex vivo* in PBMC of HLA-typed donors using an ELIspot assay (chapter 2). The most immunodominant CTL epitope of influenza A virus was the HLA-A*0201-restricted $M1_{58-66}$ epitope, since the average precursor frequency of CTL specific this epitope, was greater than the frequency of CTL specific for any other known influenza A virus epitope. The second most dominantly recognized influenza A

virus epitope was an HLA-B*2705-restricted ($NP_{383-391}$) epitope. Among the least well recognized influenza A virus CTL epitopes were the HLA-A1-restricted $PB1_{591-599}$ epitope, the HLA-A3-restricted $NP_{265-273}$ epitope and the HLA-B35-restricted $M1_{128-135}$ epitope. After *in vitro* stimulation of PBMC with influenza A virus, $M1_{58-66}$ and $NP_{383-391}$ pulsed autologous BLCL were preferentially recognized, while $PB1_{591-599}$ and $M1_{128-135}$ pulsed BLCL were not. These data confirmed the immunodominance hierarchies observed in PBMC *ex vivo*.
HLA class I background had an effect on the magnitude of epitope-specific CTL as was demonstrated in our cohort of donors. The precursor frequency of CTL specific for the HLA-B8-restricted $NP_{380-388}$ epitope was lower in PBMC of HLA-B*2705 positive donors (group II) than in PBMC of HLA-B*2705-negative donors (group I and III). This difference may have been caused by competition for presentation between the HLA-B8-restricted $NP_{380-388}$ epitope and the overlapping HLA-B*2705-restricted $NP_{383-391}$ epitope, as has been suggested previously (72). Also a greater frequency of CTL specific for the HLA-A1-restricted NP_{44-52} epitope was observed in PBMC of donors in group I. The mechanism behind this remains to be elucidated. Variation in epitope-specific precursor frequencies as a result of HLA class I background has recently been confirmed in humans infected with EBV (40).
In addition to CTL responses specific for selected epitopes, we studied the whole influenza virus-specific CTL response restricted by individual HLA-A or –B alleles (chapter 3). In PBMC of the HLA-typed donors, stimulated with influenza A virus, the majority of virus-specific CTL recognized epitopes presented by HLA-B*2705 or HLA-B*3501, followed by HLA-A*0201 or HLA-A*0301. Only a very small proportion of virus-specific CTL in the effector cell population, recognized HLA-A*0101- and HLA-B*0801-restricted epitopes. The preferred usage of HLA molecules by pathogen-specific CTL has never been demonstrated before in such detail and the question was, whether this preferred usage was unique for the virus studied and/or dependent on expressed HLA alleles (6). In contrast to the influenza A virus-specific CTL response, HLA-B*0801 was the preferred allele in the influenza B virus-specific CTL response. This indicates that the available repertoire of epitopes presented within HLA class I molecules determine which alleles are dominantly recognized and which are not.
Comparison of HLA-A or –B allele-specific responses in influenza A virus-stimulated PBMC of the HLA-typed donors demonstrated that the HLA-B*0801-restricted virus-specific CTL response was lower in HLA-B8, -B27+ donors (group II, chapter 2) than in HLA-B8, -B35+ donors (group I and III, chapter 2). This is in agreement with our data based on peptide recognition and implies that there are no other immunodominant HLA-B*0801-restricted influenza A virus-specific CTL responses present in these donors. It can not be excluded that certain responses may have been overlooked by the absence of certain epitopes in the virus (Resvir-9) used for *in vitro* stimulation.
The proportion of influenza A virus-specific CTL, determined *ex vivo* in PBMC by ELIspot and after *in vitro* stimulation of PBMC by intracellular IFN-γ staining, was greater in PBMC of HLA-A2 positive donors (group I and II) than in HLA-A2 negative donors (group III). These data and those obtained previously demonstrating that the duration of viral shedding is associated with greater precursor frequencies of

virus-specific CTL in the blood (51), suggest that HLA-A2-positive individuals are more resistant to influenza A virus infections than HLA-A2-negative individuals. Thus the association of vaccine-induced antibody titers with HLA class II haplotype (28), and the association of the magnitude of CTL response and HLA class I haplotype indicate that susceptibility to influenza A virus infection may be genetically predisposed. Further studies are required to demonstrate that indeed HLA-A2-restricted CTL responses are superior in controlling influenza A virus infections. It is tempting to speculate that a small increase in survival-rate of young HLA-A2 positive individuals during the 1918 influenza A virus pandemic has increased the HLA-A2 prevalence in Europe.
For various other pathogens, a correlation between disease and HLA class I haplotype has been identified (15, 38, 68, 77), ranging from HLA-B53 associated protection against malaria (38) to more severe complications upon secondary infections with dengue virus (68).
During the development of candidate vaccines aiming at the induction of CTL-mediated immunity the preferred HLA usage and immunodominance of specific CTL responses is not sufficiently appreciated. Furthermore, it is not always clear which epitopes induce protective CTL. For example, it was shown that a subdominant epitope in the NP induced better protection against LCMV-challenge than an immunodominant epitope on the glycoprotein (27). Suppression of CTL responses directed to certain epitopes by responses to other epitopes also have been described in the SIV model. Vaccination with a poxvector expressing SIV-Gag, resulted in reduced CTL responses to Rev and Tat proteins upon challenge of macaques with SIV, compared to control monkeys or Rev-Tat vaccinated animals (69). Since CTL against the early regulatory proteins, Rev and Tat, are inversely correlated with rapid progression to AIDS (73), vaccination with MVA-Gag could be counterproductive.

Functional diversity of influenza virus-specific CTL

Stimulation of CD8+ CTL populations with C1R cells expressing different HLA-A or –B alleles revealed differential cytokine expression in the CD8+ T cells dependent on the HLA restriction elements (chapter 3). In the HLA-A1-restricted CTL population more TNF-α+ than IFN-γ+ cells were observed in comparison with CTL populations restricted by other alleles, in particular HLA-B*0801 and HLA-B*3501. To further address functional diversity of CD8+ CTL specific for different epitopes, tetramers may be used to quantify these cells, without the need of a functional parameter. It is of interest to note that also in mice diverse cytokine expression profiles in influenza virus-specific CTL have been observed depending on the epitopes that were recognized (8). Tetramers have already been applied to identify differences in perforin and surface marker staining in CTL effector cell populations specific for EBV, CMV and HIV in the same individual (1, 2). These studies also showed a lower lytic capacity of two HIV tetramer positive cell populations in comparison with a CMV tetramer positive population. Recently it was suggested that functional differences in virus-specific CTL may be involved in establishing immunodominance patterns (65). The reason for this is unknown but

may involve premature killing of APC by superior CTL. In addition to differences in cytokine expression profiles dependent on the HLA-A and –B alleles, HLA dependent differences were found in the capacity of CTL to lyse their targets. Further studies are required to confirm these functional characteristics of CTL restricted by specific HLA alleles.
Diversity in expression profiles of cytokines, perforin and granzyme in virus-specific CTL, further complicates the analysis of virus-specific CTL responses induced by natural infection or by vaccines aiming at the induction of CTL.

CTL-mediated immune pressure on the HLA-B35-restricted $NP_{418-426}$ epitope

The importance of CTL in controlling an influenza A virus infection is underlined by the observation of amino acid variation in HLA-B*0801- and HLA-B*2705-restricted CTL epitopes associated with escape from CTL immunity (64, 74). In chapter 4 and 5 of this thesis, the identification of a new HLA-B35-restricted CTL epitope ($NP_{418-426}$) is described, which exhibited amino acid variation at non-anchor residues. The variant epitopes emerged in chronological order and CTL directed against old variants failed to recognize more recent strains of influenza A virus, indicating an escape from CTL immunity. The variation in HLA-B27- and HLA-B35-restricted epitopes correlates with the large contribution of these two alleles to the influenza A virus-specific CTL response (chapter 3), explaining the immune pressure exercised by these CTL on these epitopes. The weak CTL response restricted by ancestral haplotypes HLA-A1 and HLA-B8 (21) could imply that immunodominant epitopes presented in the context of these alleles have changed a long time ago as a result of CTL-mediated immune pressure.
So far only three of all known influenza A virus-specific CTL epitopes have been associated with escape from CTL-mediated immune pressure. The use of old reference strains, like A/Puerto Rico/8/34 and A/Hong Kong/1/68 in the past may have created a bias towards the generation of CTL clones specific for conserved epitopes. Factors like, host-restriction, packaging or functional constraints may be responsible for the conserved nature of these epitopes. Studies using PBMC isolated many years ago in combination with old and recent strains of influenza A virus may identify many more CTL epitopes in which amino acid mutations are associated with escape from CTL.
Amino acid mutations associated with escape from CTL-mediated immunity may prolong the duration of viral shedding. This may offer a better opportunity for antibody escape mutant viruses to emerge (personal communication with B. Grenfell). Finally, individuals infected with an escape mutant virus may be more susceptible to the virus due to a reduced and delayed virus-specific CTL response. *In vitro* models using PBMC of HLA-B*2705+ donors, showed that the magnitude of the virus-specific CTL response against escape mutant viruses is reduced (data not shown, EGM Berkhoff). Computer modeling has indicated that despite only 8% of the population is HLA-B27+, a mutant influenza virus lacking an HLA-B27-restricted immunodominant epitope can reach rapid fixation in the human population (31).

The effect of amino acid mutations, associated with escape from virus-specific CTL, on disease severity and immunity to the virus has been investigated in mouse models (75). Mice infected twice with a virus lacking one immunodominant CTL epitope were more susceptible to reinfection than mice infected twice with a control virus, implying that CTL-escape mutant viruses have an advantage upon reinfection. The advantage was lost if mice were reinfected with a virus lacking both immunodominant CTL epitopes. It was speculated that the reduced killing of virus-infected antigen-presenting cells gave rise to high levels of virus-specific antibody, rendering these mice less susceptible to reinfection (75).

Recognition of variant $NP_{418\text{-}426}$ epitopes by $NP_{418\text{-}426}$-specific CTL

In chapter 5, the effect of amino acid mutations within the HLA-B35-restricted $NP_{418\text{-}426}$ epitope on TCR recognition was studied. Using 11 naturally occurring variants of the epitope and 9 different $NP_{418\text{-}426}$-specific CTL clones, the concentration at which 50% of the target cells were lysed (EC_{50} value, (54)) was determined. Based on the difference in EC_{50} values of two peptides, differing at a single position in the epitope, position 4 and 5 were identified as the major determinants of TCR recognition, while position 8 was not. The importance of position 4 was shown previously for recognition of an HLA-B35-restricted CTL epitope by dengue virus-specific CTL (81).
The EC_{50} values in combination with mathematical techniques (multidimensional scaling) were used to construct an antigenic map visualizing the ability of CTL clones to recognize the variant peptides. The distance between a variant epitope and a CTL clone is a measure for the functional avidity of the CTL clone for the epitope. The distance between two variant epitopes can be used as a measure for antigenic relatedness. For example, the distance between epitopes that varied at position 4 was greater than between epitopes that varied at position 8.
The ability of CTL to recognize variant $NP_{418\text{-}426}$ epitopes was also studied, using tetramers containing these variant epitopes. The presence of T cells that recognized multiple variants was hypothesized to correlate with sequential infections with viruses containing different variants of the epitope. In mice it has already been shown that sequential infections with two influenza A viruses, containing different variants of the same epitope, induced CTL reactive against both epitopes (33). In some of the donors we found evidence supporting the induction of cross-reactive CTL upon a second infection with a virus containing a variant epitope. Since it is impossible to determine a person's infection history with 100% certainty experimental infections could be performed in HLA-B35+ individuals with a virus containing a variant $NP_{418\text{-}426}$ epitope. The induction of CTL able to recognize the natural and the variant epitope would demonstrate that consecutive infections with variant influenza A viruses will select for cross-reactive $NP_{418\text{-}426}$ specific CTL.
The ability of epitope-specific CTL clones to recognize multiple variants of a single epitope (14, 81) may be a mechanism of the immune system to counteract amino acid variation at non-anchor residues in CTL epitopes. Together with the many different T cells stimulated upon primary infection, these mechanisms will limit the

number of different epitopes that provide true escape from pre-existing CTL immunity.

Reduced immune responsiveness in the elderly

The elderly are at higher risk for developing complications related to infections with respiratory syncytial virus and influenza virus, resulting in higher morbidity and mortality rates (5, 25, 32). In the Netherlands alone, 1000-4000 excess mortality among the elderly can primarily be attributed to an infection with influenza viruses (63). The more severe clinical course of infections in the elderly is associated with a reduced immune responsiveness, also called immunosenescence. Immunosenescence has been associated with lower T cell proliferative responses and IL-2 production. In addition CTL-mediated immunity, both *in vivo* (30) and *ex vivo* (50, 60, 61), is reduced in the elderly. CTL activity is determined by a combination of several factors including precursor frequency of virus-specific CTL, proliferative capacity and effector functions per cell. In this study (chapter 6) all three parameters were investigated.
Intracellular IFN-γ staining in CD8+ T cells demonstrated that the precursor frequency of influenza A virus-specific CTL in young and old donors was comparable. This finding has been confirmed by others (45) and suggest that the elderly are capable of mounting a CTL response upon infection with influenza A virus. Previous studies that determined IFN-γ production with ELISA systems after 2-3 days of stimulation with influenza virus, did not account for differences in proliferation of lymphocytes between young and old donors, which may have skewed their data towards reduced IFN-γ responses in aging donors (9, 16, 49, 56).
In contrast to previous studies, CTL activity of effector cells from elderly donors was only slightly lower than that of young donors. Addition of rIL-2 to the *in vitro* cultures to promote T cell proliferation (36, 37), and depletion of CD4+ T- and NK-cells prior to the lysis assay, may have caused the difference in study outcome. As a result of these procedures CTL activity as a function of cytolytic effector function per cell was studied, instead of CTL activity as a function of frequency of virus-specific CTL in the effector cell population. The lower effector cell function of CTL obtained from elderly individuals may have been caused by a reduced expression of perforin and granzyme (66). Finally it was shown that the proliferative capacity of T cells of older donors after stimulation with influenza A virus was significantly reduced. These data imply that the reduced CTL activity of PBMC from elderly donors is for the most part caused by a reduction in proliferative capacity.

The effect of bioactive compounds on influenza virus-specific immune response

Malnutrition and low levels of micronutrients in the blood reduce immune activity in humans (42, 43, 48). Because of inadequate intake of one or more micronutrients by 30% of elderly individuals (23), in combination with biochemical deficiencies (34), it was thought that the reduced immune responsiveness in the elderly was caused by poor nutrition. Several studies have indicated that improving nutritional status has a

positive effect on immune function in elderly individuals (11, 17, 59). Some of these studies have used vaccination against influenza A virus, in individuals with or without food additives in their diet, to investigate the effect of bioactive compounds on immune function (18, 26, 29, 35, 62, 76). To justify these laborious and expensive field-trails, immune-enhancing effects of bioactive compounds must be evaluated first in *in vitro* and *in vivo* practical model systems.
Twenty-five different bioactive compounds were selected based on previously reported immune-enhancing properties, ranging from increased antibody production to enhanced cell mediated immunity and HLA molecule expression (4, 20, 41, 47, 70). One compound, N-acetyl-L-cysteine (NAC) enhanced antigen-specific T cell proliferation and IFN-γ production in PBMC stimulated with influenza A virus (chapter 7). Furthermore, NAC increased the lytic capacity of two influenza A virus-specific CTL clones. These and other immune-enhancing properties of NAC are thought to be caused by enhanced conjugate formation between effector and target cell (46). The concentration at which NAC increases immunological parameters is obtainable *in vivo* (39) and it was demonstrated that NAC reduces symptomatology and increases cell-mediated immune responses after influenza virus infection in the elderly (22). Prevention of apoptosis and promotion of cell survival (79) by NAC could be the mechanism behind the increased cell-mediated immune response, since influenza virus is known to induce apoptosis in immune cells (53).
Addition of chlorogenic acid to *in vitro* stimulated PBMC increased virus-induced proliferation of T cells but not IFN-γ production of CTL effector cell function. Although high concentrations of chlorogenic acid are present in eggplant and apples (44, 57), upon digestion it is rapidly metabolized into hippuric acid (55), reducing potential *in vivo* immune-enhancing effects of this compound.
The remaining 23 bioactive compounds did not enhance antigen-specific proliferation of T cells, nor the *in vitro* production of virus-specific antibodies. Although many of the compounds were known to reduce immune reactivity, the absence of immune-enhancing properties of certain vitamins, like vitamin E, was puzzling. Factors including nutrient levels in the human serum, antigen-specific proliferation and age of the donors may be responsible for this.

Conclusion

Collectively, the data presented in this thesis have shown that multiple factors influence the outcome of virus-specific CTL responses. First of all, the "pas de deux" of available immunodominant epitopes and the epitope presenting HLA molecules seems crucial in determining the magnitude and specificity of the CTL response. This dependence on HLA haplotypes should be taken into account in the development of vaccines aiming at the induction of CTL. The emergence of viruses with mutations in immunodominant epitopes is another concern regarding the induction of CTL immunity. However, the induction of cross-reactive CTL as was shown in chapter 5 may limit this potential problem. Finally, a functional diversity was observed in CD8+ T cells which was dependent on the peptide-HLA class I complexes recognized. This and the impaired functionality of CTL during aging

further complicate the evaluation of CTL immunity induced by natural infection or vaccines.

References

1. **Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael, and S. L. Rowland-Jones.** 2002. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med. **8:**379-85.
2. **Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael, and S. L. Rowland-Jones.** 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med. **192:**63-75.
3. **Badovinac, V. P., A. R. Tvinnereim, and J. T. Harty.** 2000. Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. Science. **290:**1354-8.
4. **Barbieri, B., B. Lund, B. Lundstrom, and F. Scaglione.** 1999. Coenzyme Q10 administration increases antibody titer in hepatitis B vaccinated volunteers--a single blind placebo-controlled and randomized clinical study. Biofactors. **9:**351-7.
5. **Barker, W. H., and J. P. Mullooly.** 1980. Impact of epidemic type A influenza in a defined adult population. Am J Epidemiol. **112:**798-811.
6. **Battaglia, M., and J. Gorski.** 2002. Evidence for preferred MHC class II-TCR recognition independent of the source of bound peptide. Eur J Immunol. **32:**2179-87.
7. **Belz, G. T., P. G. Stevenson, and P. C. Doherty.** 2000. Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. J Immunol. **165:**2404-9.
8. **Belz, G. T., W. Xie, and P. C. Doherty.** 2001. Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. J Immunol. **166:**4627-33.
9. **Bernstein, E. D., E. M. Gardner, E. Abrutyn, P. Gross, and D. M. Murasko.** 1998. Cytokine production after influenza vaccination in a healthy elderly population. Vaccine. **16:**1722-1731.
10. **Blattman, J. N., D. J. D. Sourdive, K. Murali-Krishna, R. Ahmed, and J. D. Altman.** 2000. Evolution of the T Cell Repertoire During Primary, Memory, and Recall Responses to Viral Infection. J Immunol. **165:**6081-6090.
11. **Bogden, J. D., J. M. Oleske, M. A. Lavenhar, E. M. Munves, F. W. Kemp, K. S. Bruening, K. J. Holding, T. N. Denny, M. A. Guarino, and B. K. Holland.** 1990. Effects of one year of supplementation with zinc and other micronutrients on cellular immunity in the elderly. J Am Coll Nutr. **9:**214-25.
12. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. The magnitude

and specificity of influenza A virus-specific cytotoxic T lymphocyte responses in humans is related to HLA-A and -B phenotype. J Virol. **76**:582-90.
13. **Bousso, P., F. Lemaitre, J. Bilsborough, and P. Kourilsky.** 2000. Facing two T cell epitopes: a degree of randomness in the primary response is lost upon secondary immunization. J Immunol. **165**:760-7.
14. **Buseyne, F., G. Janvier, B. Fleury, D. Schmidt, and Y. Riviere.** 1994. Multispecific and heterogeneous recognition of the gag protein by cytotoxic T lymphocytes (CTL) from HIV-infected patients: factors other than the MHC control the epitopic specificities. Clin Exp Immunol. **97**:353-60.
15. **Carrington, M., and S. J. O'Brien.** 2003. The influence of HLA genotype on AIDS. Annu Rev Med. **54**:535-51.
16. **Castle, S. C.** 2000. Clinical relevance of age-related immune dysfunction. Clin Infect Dis. **31**:578-585.
17. **Chandra, R. K.** 1992. Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. Lancet. **340**:1124-7.
18. **Chandra, R. K., and S. Puri.** 1985. Nutritional support improves antibody response to influenza virus vaccine in the elderly. Br Med J (Clin Res Ed). **291**:705-6.
19. **Chen, W., C. C. Norbury, Y. Cho, J. W. Yewdell, and J. R. Bennink.** 2001. Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. J Exp Med. **193**:1319-26.
20. **Chew, B. P., J. S. Park, T. S. Wong, H. W. Kim, B. B. Weng, K. M. Byrne, M. G. Hayek, and G. A. Reinhart.** 2000. Dietary beta-carotene stimulates cell-mediated and humoral immune response in dogs. J Nutr. **130**:1910-3.
21. **Dawkins, R., C. Leelayuwat, S. Gaudieri, G. Tay, J. Hui, S. Cattley, P. Martinez, and J. Kulski.** 1999. Genomics of the major histocompatibility complex: haplotypes, duplication, retroviruses and disease. Immunol Rev. **167**:275-304.
22. **De Flora, S., C. Grassi, and L. Carati.** 1997. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. European Respiratory Journal. **10**:1535-41.
23. **de Groot, C. P., T. van den Broek, and W. van Staveren.** 1999. Energy intake and micronutrient intake in elderly Europeans: seeking the minimum requirement in the SENECA study. Age Ageing. **28**:469-74.
24. **Doherty, P. C., W. E. Biddison, J. R. Bennink, and B. B. Knowles.** 1978. Cytotoxic T cell responses in mice infected with influenza and vaccinia viruses vary in magnitude with H-2 genotype. J Exp Med. **148**:534-43.
25. **Falsey, A. R., C. K. Cunningham, W. H. Barker, R. W. Kouides, J. B. Yuen, M. Menegus, L. B. Weiner, C. A. Bonville, and R. F. Betts.** 1995. Respiratory syncytial virus and influenza A infections in the hospitalized elderly. J Infect Dis. **172**:389-394.
26. **Fulop, T., Jr., J. R. Wagner, A. Khalil, J. Weber, L. Trottier, and H. Payette.** 1999. Relationship between the response to influenza vaccination and the nutritional status in institutionalized elderly subjects. J Gerontol A Biol Sci Med Sci. **54**:M59-64.

27. **Gallimore, A., T. Dumrese, H. Hengartner, R. M. Zinkernagel, and H. G. Rammensee.** 1998. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. J Exp Med. **187:**1647-57.
28. **Gelder, C. M., R. Lambkin, K. W. Hart, D. Fleming, O. M. Williams, M. Bunce, K. I. Welsh, S. E. Marshall, and J. Oxford.** 2002. Associations between human leukocyte antigens and nonresponsiveness to influenza vaccine. J Infect Dis. **185:**114-7.
29. **Girodon, F., P. Galan, A. L. Monget, M. C. Boutron-Ruault, P. Brunet-Lecomte, P. Preziosi, J. Arnaud, J. C. Manuguerra, and S. Herchberg.** 1999. Impact of trace elements and vitamin supplementation on immunity and infections in institutionalized elderly patients: a randomized controlled trial. MIN. VIT. AOX. geriatric network. Arch Intern Med. **159:**748-54.
30. **Globerson, A., and R. B. Effros.** 2000. Ageing of lymphocytes and lymphocytes in the aged. Immunol Today. **21:**515-521.
31. **Gog, J. R., G. F. Rimmelzwaan, A. D. Osterhaus, and B. T. Grenfell.** 2003. Population dynamics of rapid fixation in CTL escape mutants of influenza A virus. submitted for publication.
32. **Gross, P. A., M. Rodstein, J. R. LaMontagne, R. A. Kaslow, A. J. Saah, S. Wallenstein, R. Neufeld, C. Denning, P. Gaerlan, and G. V. Quinnan.** 1988. Epidemiology of acute respiratory illness during an influenza outbreak in a nursing home. A prospective study. Arch Intern Med. **148:**559-561.
33. **Haanen, J. B., M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher.** 1999. Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. J Exp Med. **190:**1319-1328.
34. **Haller, J.** 1999. The vitamin status and its adequacy in the elderly: an international overview. Int J Vitam Nutr Res. **69:**160-8.
35. **Hanekom, W. A., R. Yogev, L. M. Heald, K. M. Edwards, G. D. Hussey, and E. G. Chadwick.** 2000. Effect of vitamin A therapy on serologic responses and viral load changes after influenza vaccination in children infected with the human immunodeficiency virus. J Pediatr. **136:**550-2.
36. **Haynes, L., S. M. Eaton, and S. L. Swain.** 2000. The defects in effector generation associated with aging can be reversed by addition of IL-2 but not other related gamma(c)-receptor binding cytokines. Vaccine. **18:**1649-1653.
37. **Haynes, L., P. J. Linton, S. M. Eaton, S. L. Tonkonogy, and S. L. Swain.** 1999. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. J Exp Med. **190:**1013-1024.
38. **Hill, A. V., J. Elvin, A. C. Willis, M. Aidoo, C. E. Allsopp, F. M. Gotch, X. M. Gao, M. Takiguchi, B. M. Greenwood, A. R. Townsend, and et al.** 1992. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. Nature. **360:**434-9.
39. **Holdiness, M. R.** 1991. Clinical pharmacokinetics of N-acetylcysteine. Clinical Pharmacokinetics. **20:**123-34.
40. **Hollsberg, P.** 2002. Contribution of HLA class I allele expression to CD8+ T cell responses against Epstein-Barr virus. Scand J Immunol. **55:**189-95.

41. **Jyonouchi, H., S. Sun, M. Mizokami, and M. D. Gross.** 1996. Effects of various carotenoids on cloned, effector-stage T-helper cell activity. Nutrition and Cancer. **26:**313-24.
42. **Lesourd, B., and L. Mazari.** 1999. Nutrition and immunity in the elderly. Proc Nutr Soc. **58:**685-95.
43. **Lesourd, B. M., L. Mazari, and M. Ferry.** 1998. The role of nutrition in immunity in the aged. Nutr Rev. **56:**S113-125.
44. **Lo, H. H., and J. G. Chung.** 1999. The effects of plant phenolics, caffeic acid, chlorogenic acid and ferulic acid on arylamine N-acetyltransferase activities in human gastrointestinal microflora. Anticancer Research. **19:**133-9.
45. **Looney, R. J., A. R. Falsey, E. Walsh, and D. Campbell.** 2002. Effect of aging on cytokine production in response to respiratory syncytial virus infection. J Infect Dis. **185:**682-5.
46. **Malorni, W., A. D'Ambrosio, G. Rainaldi, R. Rivabene, and M. Viora.** 1994. Thiol supplier N-acetylcysteine enhances conjugate formation between natural killer cells and K562 or U937 targets but increases the lytic function only against the latter. Immunology Letters. **43:**209-14.
47. **Marshall, M. E., J. L. Rhoades, C. Mattingly, and C. D. Jennings.** 1991. Coumarin (1,2-benzopyrone) enhances DR and DQ antigen expressions by peripheral blood mononuclear cells in vitro. Mol Biother. **3:**204-6.
48. **Mazari, L., and B. M. Lesourd.** 1998. Nutritional influences on immune response in healthy aged persons. Mech Ageing Dev. **104:**25-40.
49. **Mbawuike, I. N., C. L. Acuna, K. C. Walz, R. L. Atmar, S. B. Greenberg, and R. B. Couch.** 1997. Cytokines and impaired CD8+ CTL activity among elderly persons and the enhancing effect of IL-12. Mech Ageing Dev. **94:**25-39.
50. **Mbawuike, I. N., A. R. Lange, and R. B. Couch.** 1993. Diminished influenza A virus-specific MHC class I-restricted cytotoxic T lymphocyte activity among elderly persons. Viral Immunol. **6:**55-64.
51. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare.** 1983. Cytotoxic T cell immunity to influenza. N Engl J Med. **309:**13-17.
52. **Mo, A. X., S. F. van Lelyveld, A. Craiu, and K. L. Rock.** 2000. Sequences that flank subdominant and cryptic epitopes influence the proteolytic generation of MHC class I-presented peptides. J Immunol. **164:**4003-10.
53. **Nichols, J. E., J. A. Niles, and N. J. Roberts, Jr.** 2001. Human lymphocyte apoptosis after exposure to influenza A virus. J Virol. **75:**5921-5929.
54. **O'Connor, D. H., T. M. Allen, T. U. Vogel, P. Jing, I. P. DeSouza, E. Dodds, E. J. Dunphy, C. Melsaether, B. Mothe, H. Yamamoto, H. Horton, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat Med. **8:**493-9.
55. **Olthof, M. R., P. C. Hollman, M. N. Buijsman, J. M. Van Amelsvoort, and M. B. Katan.** 2003. Chlorogenic Acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. J Nutr. **133:**1806-14.
56. **Ouyang, Q., G. Cicek, R. G. Westendorp, H. J. Cools, R. J. van der Klis, and E. J. Remarque.** 2000. Reduced IFN-gamma production in elderly people

following in vitro stimulation with influenza vaccine and endotoxin. Mech Ageing Dev. **121:**131-137.

57. **Paganga, G., N. Miller, and C. A. Rice-Evans.** 1999. The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute? Free Radical Research. **30:**153-62.

58. **Pawelec, G., Y. Barnett, R. Forsey, D. Frasca, A. Globerson, J. McLeod, C. Caruso, C. Franceschi, T. Fulop, S. Gupta, E. Mariani, E. Mocchegiani, and R. Solana.** 2002. T cells and aging, January 2002 update. Front Biosci. **7:**d1056-183.

59. **Penn, N. D., L. Purkins, J. Kelleher, R. V. Heatley, B. H. Mascie-Taylor, and P. W. Belfield.** 1991. The effect of dietary supplementation with vitamins A, C and E on cell- mediated immune function in elderly long-stay patients: a randomized controlled trial. Age Ageing. **20:**169-74.

60. **Powers, D. C.** 1993. Influenza A virus-specific cytotoxic T lymphocyte activity declines with advancing age. J Am Geriatr Soc. **41:**1-5.

61. **Powers, D. C., and R. B. Belshe.** 1993. Effect of age on cytotoxic T lymphocyte memory as well as serum and local antibody responses elicited by inactivated influenza virus vaccine. J Infect Dis. **167:**584-592.

62. **Provinciali, M., A. Montenovo, G. Di Stefano, M. Colombo, L. Daghetta, M. Cairati, C. Veroni, R. Cassino, F. Della Torre, and N. Fabris.** 1998. Effect of zinc or zinc plus arginine supplementation on antibody titre and lymphocyte subsets after influenza vaccination in elderly subjects: a randomized controlled trial. Age Ageing. **27:**715-22.

63. **Remarque, E. J.** 1999. Influenza vaccination in elderly people. Exp Gerontol. **34:**445-52.

64. **Rimmelzwaan, G. F., A. C. Boon, J. T. Voeten, E. G. Berkhoff, R. A. Fouchier, and A. D. Osterhaus.** 2003. Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. In press in Virus research.

65. **Roy-Proulx, G., M. C. Meunier, A. M. Lanteigne, S. Brochu, and C. Perreault.** 2001. Immunodomination results from functional differences between competing CTL. Eur J Immunol. **31:**2284-92.

66. **Rukavina, D., G. Laskarin, G. Rubesa, N. Strbo, I. Bedenicki, D. Manestar, M. Glavas, S. E. Christmas, and E. R. Podack.** 1998. Age-related decline of perforin expression in human cytotoxic T lymphocytes and natural killer cells. Blood. **92:**2410-2420.

67. **Schipper, R. F., G. M. Schreuder, J. D'Amaro, and M. Oudshoorn.** 1996. HLA gene and haplotype frequencies in Dutch blood donors. Tissue Antigens. **48:**562-74.

68. **Stephens, H. A., R. Klaythong, M. Sirikong, D. W. Vaughn, S. Green, S. Kalayanarooj, T. P. Endy, D. H. Libraty, A. Nisalak, B. L. Innis, A. L. Rothman, F. A. Ennis, and D. Chandanayingyong.** 2002. HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. Tissue Antigens. **60:**309-18.

69. **Stittelaar, K. J., R. A. Gruters, M. Schutten, C. A. van Baalen, G. van Amerongen, M. Cranage, P. Liljestrom, G. Sutter, and A. D. Osterhaus.** 2002.

Comparison of the efficacy of early versus late viral proteins in vaccination against SIV. Vaccine. **20:**2921-7.
70. **Tatefuji, T., N. Izumi, T. Ohta, S. Arai, M. Ikeda, and M. Kurimoto.** 1996. Isolation and identification of compounds from Brazilian propolis which enhance macrophage spreading and mobility. Biol Pharm Bull. **19:**966-70.
71. **Tourdot, S., and K. G. Gould.** 2002. Competition between MHC class I alleles for cell surface expression alters CTL responses to influenza A virus. J Immunol. **169:**5615-21.
72. **Tussey, L. G., S. Rowland-Jones, T. S. Zheng, M. J. Androlewicz, P. Cresswell, J. A. Frelinger, and A. J. McMichael.** 1995. Different MHC class I alleles compete for presentation of overlapping viral epitopes. Immunity. **3:**65-77.
73. **van Baalen, C. A., O. Pontesilli, R. C. Huisman, A. M. Geretti, M. R. Klein, F. de Wolf, F. Miedema, R. A. Gruters, and A. D. Osterhaus.** 1997. Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. J Gen Virol. **78:**1913-8.
74. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J Virol. **74:**6800-6807.
75. **Webby, R. J., S. Andreansky, J. Stambas, J. E. Rehg, R. G. Webster, P. C. Doherty, and S. J. Turner.** 2003. Protection and compensation in the influenza virus-specific CD8+ T cell response. Proc Natl Acad Sci U S A. **100:**7235-7240.
76. **Wouters-Wesseling, W., M. Rozendaal, M. Snijder, Y. Graus, G. Rimmelzwaan, L. De Groot, and J. Bindels.** 2002. Effect of a complete nutritional supplement on antibody response to influenza vaccine in elderly people. J Gerontol A Biol Sci Med Sci. **57:**M563-6.
77. **Yenigun, A., and B. Durupinar.** 2002. Decreased frequency of the HLA-DRB1*11 allele in patients with chronic hepatitis C virus infection. J Virol. **76:**1787-9.
78. **Yewdell, J. W., and J. R. Bennink.** 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu Rev Immunol. **17:**51-88.
79. **Zafarullah, M., W. Q. Li, J. Sylvester, and M. Ahmad.** 2003. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci. **60:**6-20.
80. **Zinkernagel, R. M., and P. C. Doherty.** 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T cell restriction-specificity, function, and responsiveness. Adv Immunol. **27:**51-177.
81. **Zivny, J., I. Kurane, A. M. Leporati, M. Ibe, M. Takiguchi, L. L. Zeng, M. A. Brinton, and F. A. Ennis.** 1995. A single nine-amino acid peptide induces virus-specific, CD8+ human cytotoxic T lymphocyte clones of heterogeneous serotype specificities. J Exp Med. **182:**853-63.

Nederlandse Samenvatting

Een belangrijke functie van het immuunsysteem is het verdedigen van het lichaam tegen virussen, bacteriën, schimmels en parasieten. Naast een niet-specifieke arm van het immuunsysteem bestaat er ook een specifieke arm die gericht lichaamsvreemde componenten kan herkennen en aanvallen.
Om een virusinfectie te bestrijden, ontwikkelt het lichaam virusspecifieke witte bloedcellen zgn B- en T-cellen. B-cellen produceren antistoffen welke het virus direct kunnen neutraliseren terwijl T-cellen en dan met name cytotoxische T-lymfocyten (CTL) de duur van de infectie kunnen verkorten. Dit is mogelijk doordat CTL met virus geïnfecteerde cellen opruimen waardoor de aanmaak van nieuwe virussen wordt gereduceerd. Hoe sneller dit gebeurt, hoe korter de duur van de infectie en daardoor de duur van de ziekte. CTL kunnen onderscheid maken tussen met virus geïnfecteerde cellen en normale cellen doordat zij kleine stukjes van virale eiwitten (epitopen genaamd) herkennen die worden gepresenteerd door speciale moleculen aan de buitenkant van een menselijke cel. Deze moleculen worden HLA (humaan leucocyten antigeen) moleculen genoemd en zijn sterk gevarieerd. Een gegeven epitoop kan meestal door één bepaald type HLA molecuul worden gebonden en op het celoppervlak gepresenteerd; dit verschijnsel heet restrictie. Als virale epitopen worden herkend door bijbehorende specifieke CTL, dan worden de CTL geactiveerd en dit leidt tot lysis van de met virus geïnfecteerde cel en remming van de virusvermeerdering in het lichaam. Eén van de virussen waarbij specifieke immuniteit een belangrijke rol speelt, is het influenza virus. Influenzavirussen zijn de veroorzakers van de acute luchtweginfectie die beter bekend is als "influenza". Influenza kan heel heftig zijn en met name bij oudere mensen leiden tot ernstige complicaties en zelfs de dood. In de studies voor dit proefschrift is de CTL respons tegen influenza virussen nader onderzocht.

In hoofdstuk 2 wordt de respons tegen bekende CTL epitopen van influenza A virussen behandeld. Van de tien bekende CTL epitopen werden sommige frequenter herkend dan andere, een fenomeen dat immunodominantie wordt genoemd. Het meest immunodominante epitoop was $M1_{58\text{-}66}$, dat wordt gepresenteerd door HLA-A2, gevolgd door het $NP_{383\text{-}391}$ epitoop dat door HLA-B27 wordt gepresenteerd. Naast de efficiëntere herkenning van bepaalde CTL epitopen bleek ook dat het aantal CTL specifiek voor het $NP_{380\text{-}388}$ epitoop, dat wordt gepresenteerd wordt door HLA-B8, kleiner was in HLA-B27 positieve dan in HLA-B27 negatieve donoren. Ook was het aantal CTL gericht tegen het $NP_{44\text{-}51}$ epitoop, dat wordt gepresenteerd door HLA-A1, groter in een bepaalde groep donoren met identieke HLA moleculen dan in twee andere groepen donoren met identieke HLA moleculen. Deze verschillen in epitoop specifieke CTL responsen in groepen donoren met identieke HLA moleculen, duiden erop dat de hoogte van de CTL respons afhankelijk is van het HLA fenotype van een persoon. Een ander voorbeeld van een genetische factor bij de cellulaire immuunrespons is dat het totale aantal influenza A virusspecifieke CTL hoger was in HLA-A2 positieve dan in HLA-A2 negatieve donoren. Of de betere CTL respons in HLA-A2 positieve donoren ook leidt tot een kortere

ziekteduur bij influenza A in deze groep van individuen, dient nader te worden onderzocht.

Omdat veel CTL epitopen van het influenza-A-virus nog niet bekend zijn, is het op basis van de hier beschreven studies thans nog niet mogelijk om te zeggen welk HLA-molecuul, door het presenteren van immunodominante epitopen van het influenza A virus, de voorkeur heeft bij influenza A virusspecifieke CTLs. Op basis van bekende epitopen zouden we kunnen concluderen dat HLA-A2 gerestricteerde epitopen dominant zullen zijn. Om binnen de totale influenza A virusspecifieke CTL respons toch de voorkeur voor HLA moleculen met bekende en nog onbekende epitopen te kunnen bepalen, is gebruik gemaakt van cellen die slechts één type HLA molecuul tot expressie brengen. In deze cellen zijn alle bekende en onbekende CTL-epitopen voor hun presentatie afhankelijk van dit ene type HLA molecuul. Met behulp van deze cellen werd aangetoond dat het grootste deel van de influenza A virusspecifieke CTL epitopen herkennen die worden gepresenteerd door HLA-B27 of HLA-B35 (hoofdstuk 3). Ook herkent een groot deel van de CTL, HLA-A2 en HLA-A3 gerestricteerde epitopen. Het aantal CTL specifiek voor epitopen gepresenteerd door HLA-A1 en HLA-B8 bleek daarentegen klein.
Dezelfde techniek is gebruikt om te bepalen welke HLA moleculen worden herkend door influenza B virusspecifieke CTL. In tegenstelling tot CTL tegen influenza A virussen herkennen de meeste influenza B virusspecifieke CTL epitopen gepresenteerd door HLA-B8. Hieruit blijkt dat de voorkeur voor bepaalde HLA moleculen afhankelijk is van het virus ofwel de virale epitopen die worden gepresenteerd, en niet of in mindere mate van het HLA molecuul zelf.

In hoofdstuk 4 wordt een nieuw CTL-epitoop van het influenza A virus ($NP_{418-426}$) beschreven dat wordt gepresenteerd door HLA-B35. Dit epitoop wordt herkend door influenza A virusspecifieke CTL en kan één van de nog onbekende epitopen zijn die verantwoordelijk zijn voor de voorkeur van influenza A virusspecifieke CTL voor HLA-B35. Toen de aminozuursequentie van dit HLA-B35 epitoop uit influenza A virussen van nu en vroeger werden vergeleken, bleek deze sequentie in de loop van de tijd te zijn veranderd. Nader onderzoek gaf aan dat CTL gericht tegen oudere varianten van het $NP_{418-426}$ epitoop niet in staat waren om de huidige variant van het $NP_{418-426}$ epitoop te herkennen. Toen de huidige epitoopvariant ontstond, had het virus met deze mutatie dus mogelijk een voordeel ten opzichte van de andere circulerende virussen waarvan het $NP_{418-426}$ epitoop wel door de CTL werd herkend. Hiermee is een aanwijzing gevonden dat niet alleen antistof maar ook cellulaire immuniteit antigene drift van humaan influenzavirus kan bewerkstelligen.

De bovenbedoelde veranderingen in aminozuursequentie vonden plaats op posities 4, 5, 6 en 8 van het epitoop $NP_{418-426}$ (hoofdstuk 5). Het effect van elk van deze aminozuurveranderingen (mutaties) op de herkenning door $NP_{418-426}$ specifieke CTL is onderzocht. De mutaties op posities 4 en 5 hadden als gevolg dat de meeste CTL klonen het epitoop niet langer herkenden. De mutaties op posities 6 en 8 hadden weinig of geen effect op de herkenning van het epitoop door CTL klonen. Van de negen CTL klonen waarmee het effect van de verschillende mutaties werd

onderzocht, bleek er één geen onderscheid te maken tussen de verschillende varianten van het epitoop. Dat CTL meerdere varianten van hetzelfde epitoop kunnen herkennen, wijst op het vermogen van het immuunsysteem om virussen die muteren soms toch te blijven herkennen.
Omdat er in de laatste decennia voornamelijk twee influenza A virussubtypen (H1N1 en H3N2) circuleren die verschillen in hun $NP_{418-426}$ epitoop, is de herkenning van beide varianten van dit epitoop door CTL in bloed van HLA-B35+ donoren bestudeerd. In witte bloedcellen gestimuleerd met één van beide subtypen, werden CTL aangetoond die meestal één maar soms beide varianten van het epitoop konden herkennen. Omdat deze varianten in twee verschillende influenza A virussubtypen aanwezig zijn, kon er door middel van serumonderzoek worden gekeken of infecties in het verleden van de donoren met beide subtypen geassocieerd zijn met de aanwezigheid van kruisreagerende CTL die beide epitoopvarianten herkennen. Bij twee van de vijf donoren bleek dit inderdaad het geval.

Naast de hierboven beschreven meer fundamentele vraagstellingen betreffende de CTL respons tegen influenza virussen, is de CTL immuniteit tegen influenza A virus in ouderen ten opzichte van jongeren onderzocht (hoofdstuk 6). Van ouderen is bekend dat deze vaak een minder goed functionerend immuunsysteem hebben wat met name lijkt te worden veroorzaakt door functionele defecten in T-helper cellen. T-helper cellen helpen bij het tot stand komen van zowel een goede antistof- als een goede CTL respons. Als gevolg van genoemde functionele defecten zijn bij ouderen beide responsen tegen virussen verminderd. CTL activiteit wordt bepaald door kwalitatieve en kwantitatieve parameters, waaronder het totale aantal virusspecifieke CTL, de snelheid waarmee deze cellen na contact met het specifieke antigeen gaan delen en de mate waarin CTL met influenza virus geïnfecteerde cellen kunnen lyseren of anderszins in deze cellen de virusreplicatie kunnen doen afnemen. Het aantal influenza virusspecifieke T-cellen in het bloed van jonge donoren bleek niet te verschillen van dat bij oudere donoren. Wel was de capaciteit van influenza virusspecifieke CTL van jongeren om met virus geïnfecteerde cellen te lyseren beter dan die bij ouderen. Het grootste verschil dat werd gevonden tussen T-cellen van jongeren en ouderen was echter hun delingssnelheid na toevoeging van influenza virus.

Voeding kan een belangrijke rol spelen in de afweer tegen infectieuse agentia. Met name bij jonge kinderen en ouderen kunnen voedingscomponenten het goed functioneren van het immuunsysteem bevorderen. In de studies voor dit proefschrift is van een aantal bioactieve stoffen bepaald of zij de respons van influenza virusspecifieke T-cellen kunnen verbeteren. Van de 25 geteste bioactieve stoffen bleken er twee een positief effect te hebben op de delingssnelheid van witte bloedcellen na stimulatie met influenza A virus (hoofdstuk 7): toevoeging van N-acetyl-L-cysteïne aan T-cel cultures verhoogde de delingscapaciteit van deze cellen. Daarnaast gingen de T-cellen meer interferon-γ produceren en bleken virusspecifieke CTL beter in staat om met virus geïnfecteerde cellen te lyseren. De tweede stof is chlorogeenzuur, welke alleen de delingscapaciteit van witte

bloedcellen verhoogde. Deze data wijzen erop dat de twee genoemde voedingscomponenten inderdaad het functioneren van het immuunsysteem kunnen verbeteren. Vervolgstudies in muizen en klinische studies moeten aantonen of beide stoffen ook werkelijk een positief effect hebben op het reduceren van de ernst van het klinisch verloop van infecties, in het bijzonder van influenza.

Dankwoord

Nu is dan het moment aangebroken om iedereen te bedanken die heeft geholpen bij het tot standkomen van dit proefschrift. BEDANKT ALLEMAAL.

Toch wil ik een nog een aantal mensen in het bijzonder noemen. Allereerst wil ik mijn promotor Ab Osterhaus bedanken voor het in mij gestelde vertrouwen en de vrijheid die ik heb gekregen om aan allerlei "epifenomenen" te werken. Vijf jaar onderzoek aan CTLs en influenza virus hebben toch dit wetenschappelijke "middenstandsdiploma" opgeleverd.

Heel veel lof voor jou Guus. Je rust en geduld tijdens het opschrijven van de experimenten zijn heel belangrijk geweest voor het halen van de eindstreep. Onze discussies hebben ervoor gezorgd dat ik productief ben gaan denken. De mooie verhalen, biertje aan het einde van de week, barbecues bij jouw thuis, het strand van Malta en Curaçao (2x), snorkelen en Parijs hebben het promoveren onder jou begeleiding tot een hele leuke tijd gemaakt.

Gerrie, jouw hulp is van onschatbare waarde geweest. Bulkstimulaties, CTL assays, klonen en targets, bijna niets was te gek en zonder jouw harde werken was het niet gelukt om alle data te verzamelen. Verder heeft jouw drang naar vakantie mij doen beseffen dat het heel verstandig is om af en toe even een break te hebben.

Byron, jij was altijd bereid tot het delen van je kennis en onderling hebben we dan ook vaak discussies gehad over jouw en mijn onderzoek. Tiny, geheel nieuwe technieken en steeds maar doorgaan op een lastig project, petje af. My experience with Italy. Elena, I had a lot of fun during the time we spent in the lab, the many hours behind the flow cytometer, in Texel and occasionally in the pub. Femke kan ik kort samenvatten, vragen en verhalen, erg gezellig hoor. Ron, bedankt voor de kritische blik bij het lezen van de manuscripten en natuurlijk het idee voor de omslag van het boekje.

Heel veel experimenten binnen dit promotieonderzoek zouden niet mogelijk zijn geweest zonder materialen van anderen. Prof. F. Claas en dr. D. Roelen (LUMC) wil ik bedanken voor het verstrekken van historische PBMC. Dr. P. van den Bruggen voor het fluoriscerende referentie peptide, dr. D. van Baarle en G. van Schijndel (CLB) voor het synthetiseren van HLA klasse I peptide-tetrameer complexen, dr. W. Beyer voor het de statistische analyses en dr. P. Cresswell, dr. P. Romero, dr. M. Takiguchi, dr. W. Biddison, dr. J. Lopez de Castro voor het opsturen van de verschillende cellijnen. Verder wil ik ook de mensen van Numico Research bedanken voor hun bijdrage aan het proefschrift. Finally, I would like to thank dr. D. Smith and dr. A. Lapedes for their contribution to chapter 5.

Op deze plaats wil ik ook de mensen bedanken die aan het begin hebben gestaan van mijn tot nu toe korte carrière. Many thanks to dr. M. Zambon (PHLS), who allowed me to work in her lab for over a year and tought me many things about influenza

virus and epidemiology. Deze buitenlandse stage heb ik volledig te danken aan dr. B. Palache (Solvay Pharmaceuticals), die ik hiervoor veel dank verschuldigd ben.

Ook wil ik bij deze mijn huidige kamergenoten en flu collega's bedanken: Chantal, Derek, Emmie, Jan, Marianne, Monique, Miranda, Nella, Roel, Ruud, Sander, Theo B., Vincent, de studenten: Ellen en Michel, en de ex-influenza collega's: Liane en Theo V., voor de gezellige sfeer tijdens het werken; southpark poppetjes, voetballen tussen de liften, sinterklaas, borrels etc. Ook veel dank aan alle andere collegas van "de virologie" voor de noodzakelijke koffie, hulp, materialen, kennis en plezier tijdens het werken.

Dit is ook de plaats om de bloeddonoren te bedanken voor het beschikbaar stellen van hun bloed voor onderzoeksdoeleinden. Daarnaast wil ik ook de mensen van de Bloedbank Rotterdam te bedanken, Kees, Saskia, Wilfried en alle anderen die ik niet bij naam ken maar die hebben geholpen bij het zoeken, oproepen en HLA typeren van de vele donoren die noodzakelijk waren voor mijn onderzoek.

Mijn familie en vrienden wil ik bij deze ook noemen. Ouders, Pieter en Dorien, voor jullie steun aan en interesse in mijn persoontje. Gisbert, Chantal, Robert, Inez, Arnout, Nick, Richard, Michel, Marleen en alle andere vrienden voor de fantastische tijden; de Ruif, de Bijlmer, parachute springen, skiën, kermis, studie en nog vele andere mooie momenten.

Dan blijft er nog één iemand over. Jacaranda, onze gesprekken op zondagmiddag tijdens jouw verblijf in Bolivia en de romantische vakanties in Zuid-Amerika hebben ervoor gezorgd dat ik met veel enthousiasme mijn promotieonderzoek heb kunnen afronden.

Curriculum Vitae

Adrianus Cornelis Maria Boon is geboren op 9 september 1973 in Venhuizen. Na het afronden van de MAVO (Odulphus Mavo, Lutjebroek) en het VWO (Marcus College, Grootebroek) in respectievelijk 1989 en 1992, ben ik Medische Biologie gaan studeren aan de Universiteit van Amsterdam. Tijdens de studie heb ik stage gelopen binnen de vakgroep Bacteriologie van het Academisch Medisch Centrum in Amsterdam (Dr. B. Zaat). Tevens heb ik 7 maanden onderzoek gedaan bij Dr. M. Zambon in het Central Public Health Laboratory te Londen. Na het behalen van het doctoraal diploma in 1997 werd mij een baan aangeboden als clinical scientist level A binnen het lab van Dr. M. Zambon. In september 1998 ben ik begonnen als assistent in opleiding (AIO) op de afdeling Virologie van het ErasmusMC. Onder leiding van prof.dr. A.D.M.E. Osterhaus en dr. G.F. Rimmelzwaan heb ik promotieonderzoek gedaan wat leidde tot het voltooien van dit proefschrift.

Publications

W.L. Irving, D.K. James, T. Stephenson, P. Laing, C. Jameson, J.S. Oxford, P. Chakraverty, D.W.G. Brown, **A.C.M. Boon**, M.C. Zambon. 2000. Influenza virus infection in the second and third trimesters of pregnancy: a clinical and seroepidemiological study. British Journal of Obstetrics and Gynaecology. 107(10); 1282-1289

A.C.M. Boon, A.F. French, D.M. Fleming, M.C. Zambon. 2001. Detection of influenza a subtypes in community-based surveillance. Journal of Medical Virology. 65(1); 163-170

T.M. Lawson, S. Man, S. Williams, **A.C.M. Boon**, M.C. Zambon, L.K. Borysiewicz. 2001. Influenza A antigen exposure selects dominant Vβ17^{+} T cell receptors in human CD8+ T cytotoxic T cell responses. International Immunology. 13(11); 1373-81

A.C.M. Boon, P. Vos, Y.M.F. Graus, G.F. Rimmelzwaan, A.D.M.E. Osterhaus. 2002. *In vitro* effect of two dietary compounds on influenza virus-specific B and T cell responses. Scandinavian Journal of Immunology. 55(1); 24-32

A.C.M. Boon, G. de Mutsert, Y.M.F. Graus, R.A.M. Fouchier, K. Sintnicolaas, A.D.M.E. Osterhaus, G.F. Rimmelzwaan 2002. The magnitude and specificity of influenza A virus-specific CTL responses in humans is related to HLA-A and -B phenotype. Journal of Virology. 76(2); 582-90

A.C.M. Boon, G. de Mutsert, Y.M.F. Graus, R.A.M. Fouchier, K. Sintnicolaas, A.D.M.E. Osterhaus, G.F. Rimmelzwaan. 2002. Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein

associated with escape from cytotoxic T lymphocytes. Journal of Virology. 76(5); 2567-72

A.C.M. Boon, E. Fringuelli, Y.M.F. Graus, R.A.M. Fouchier, K. Sintnicolaas, A.M. Iorio, G.F. Rimmelzwaan, A.D.M.E. Osterhaus. 2002 Influenza virus A specific T cell immunity in humans during aging. Virology. 299(1); 100-8

A.C.M. Boon, G. de Mutsert, D. van Baarle, D.J. Smith, A.S. Lapedes, R.A.M. Fouchier, K. Sintnicolaas, A.D.M.E. Osterhaus, G.F. Rimmelzwaan 2003. Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes. *Submitted for publication*

A.C.M. Boon, G. de Mutsert, R.A.M. Fouchier, K. Sintnicolaas, A.D.M.E. Osterhaus, G.F. Rimmelzwaan. 2003. Preferred HLA usage and HLA dependent differential cytokine expression in the influenza virus-specific cytotoxic T lymphocyte response. *Submitted for publication.*

G.F. Rimmelzwaan, **A.C.M. Boon**, J.T.M. Voeten, E.G.M. Berkhoff, R.A.M. Fouchier, A.D.M.E. Osterhaus. Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *Submitted for publication*

G.F. Rimmelzwaan, **A.C.M. Boon**, M.M. Geelhoed-Mieras, J.T.M. Voeten, R.A.M. Fouchier, A.D.M.E. Osterhaus. 2003 Human airway epithelial cells present antigen to influenza virus-specific CD8+ CTL inefficiently after incubation with viral protein together with ISCOMATRIX®. *Submitted for publication*

J.C. de Jong, A.M. Palache, W.E.P. Beyer, G.F. Rimmelzwaan, **A.C.M. Boon**, A.D.M.E. Osterhaus. Hemagglutination-inhibiting antibody to influenza virus. *Submitted for publication*

Stellingen

Behorende bij het proefschrift
'Cytotoxic T lymphocyte-mediated immunity to influenza'

1. De mate van herkenning van virale peptiden in associatie met HLA allelen door virusspecifieke cytotoxische T-lymfocyten (CTL) is mede afhankelijk van de presentatie van virale peptiden door andere HLA allelen (*dit proefschrift*).

2. De intensiteit van de influenza A virusspecifieke CTL respons wordt mede bepaald door het HLA genotype van de gastheer (*dit proefschrift*).

3. De voorkeur voor herkenning van HLA allelen in associatie met virale peptiden door virusspecifieke CTLs is mede afhankelijk van het virustype (*dit proefschrift*).

4. Influenza A virusspecifieke CTL activiteit veroorzaakt antigene drift (*dit proefschrift*).

5. De reductie in virusspecifieke CTL activiteit bij ouderen is mede het gevolg van verminderde delingscapaciteit van T-cellen (*dit proefschrift*).

6. Partnerkeuze op basis van lichaamsgeur verhoogt de MHC diversiteit in het nageslacht (Jacobs, S. *Nature Genetics 2002 Feb;30(2):175-9*).

7. Computermodellen (Derek Smith, *Vaccine, 2003*) zullen in de toekomst bepalend zijn voor de stamkeuze voor influenzavaccins.

8. De mediahype betreffende bioterrorisme en biologische oorlogsvoering wordt misbruikt voor het rechtvaardigen van oorlogshandelingen.

9. Het Europese landbouwbeleid is een effectief massavernietigingswapen.

10. Een influenza A virus "early-warning" systeem met serologische surveillance in pluimvee en wilde vogels à één miljoen euro per jaar zou een uitbraak van vogelpest in Nederland à één miljard euro hebben voorkomen.

11. Een éénmalige investering van honderd miljoen euro aan antivirale middelen ter bestrijding van een influenza pandemie, zal veel meer mensenlevens sparen dan de vele miljarden die de komende jaren extra worden besteed aan verkeersveiligheid, brandpreventie en "blauw op straat".

Jacco Boon, Rotterdam, 17 september 2003