Influenza Virus-specific $CD8^+$ T Cells

-longevity, cross-reactivity and viral evasion-

Carolien van de Sandt

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Influenzavirus-specifieke CD8$^+$ T-cellen

-levensduur, kruis-reactiviteit en virale ontwijking-

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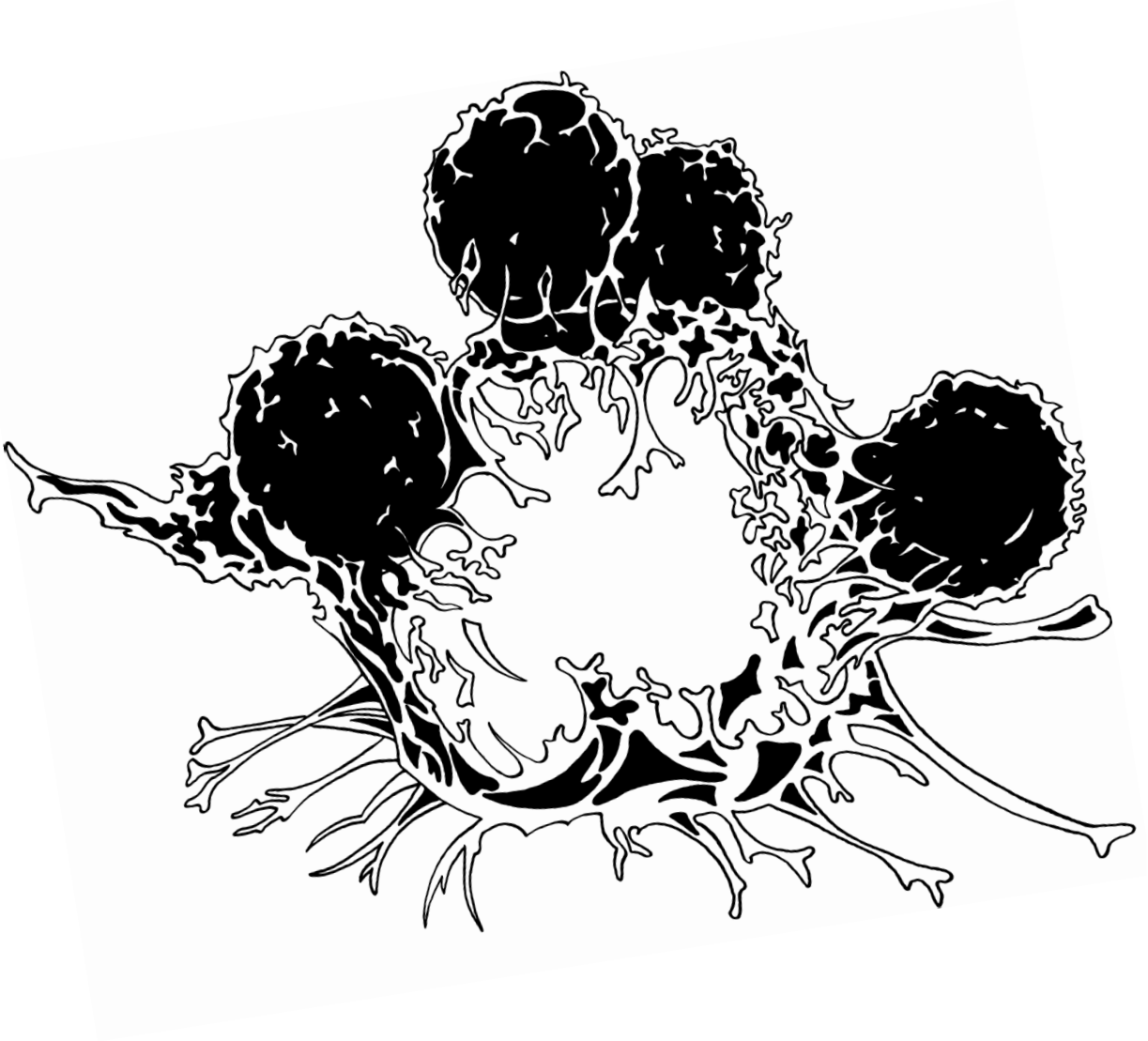
–If you never try, you will never know–

Contents



CHAPTER 1:

General introduction

Partially based on:

Evasion of influenza A viruses from innate and adaptive immune responses
Carolien E. van de Sandt, Joost H.C.M. Kreijtz and Guus F. Rimmelzwaan
Viruses, 2012, 4, 1438-1476

And

Influenza B viruses: Not to be discounted
Carolien E. van de Sandt, Rogier Bodewes, Guus F. Rimmelzwaan and Rory D. de Vries
Future Microbiology, 2015, 10, 1447-1465

Influenza A and B viruses

Influenza viruses that belong to the family *of Orthomyxoviridae,* which consists of six genera: Isavirus, Thogoto virus and influenza virus A, B, C and D. Influenza viruses have a diameter of 80-120nm and are distinguished bases on their surface glycoproteins, membrane channel proteins and their genome size [1]. Influenza A viruses are further classified based on the antigenic properties of their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Thus far, 18 HA subtypes (H1-H18) and 11 NA subtypes (N1-N11) have been identified [2-5]. Although influenza B viruses are not subdivided into subtypes, two genetically and antigenically distinct lineages can be discriminated based on the HA surface glycoprotein, namely the B/Yamagata lineage and the B/Victoria lineage [6]. The nomenclature of influenza viruses is based on the type / host of origin (except human) / isolation site (geographically) / strain number / year of isolation and is followed by the description of the antigenic subtype, e.g. A/Chicken/Netherlands/1/2003 (H7N7) or B/Netherlands/455/2011 (B/Victoria lineage).

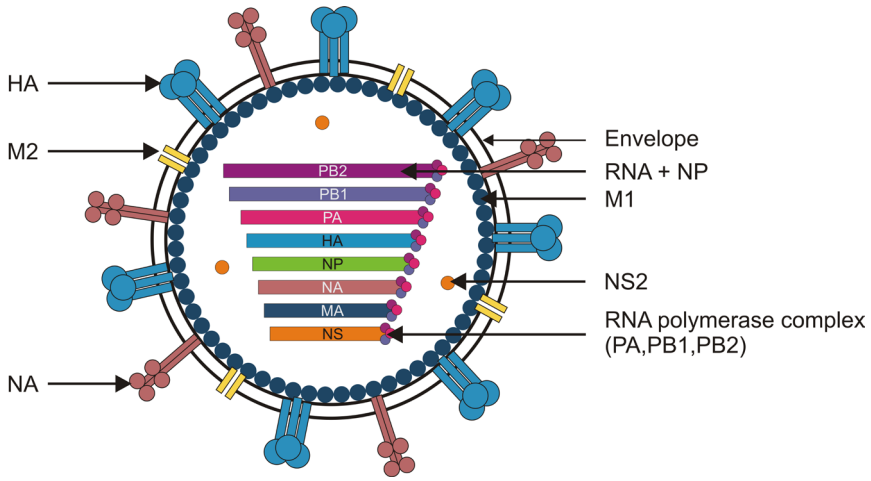


Figure 1 Schematic overview of an influenza A virus particle and gene segments

Structure and proteins of influenza A and B viruses

Influenza virus particles are enveloped and have a single stranded negative sense RNA genome that is divided over eight separate gene segments (Figure 1). The influenza A genome encodes for 17 proteins, the influenza B genome encodes for 11 proteins [2, 7] an overview of these differences in protein expression can be found in table 1. The eight RNA gene segments are encapsidated by nucleoproteins (NP), and are associated with the polymerase basic protein 1 (PB1) and PB2 and polymerase acidic protein (PA), which together form the ribonuleoprotein (RNP) complex [8, 9]. The polymerase proteins initiate replication of the viral RNA (vRNA) and transcription into messenger RNA (mRNA) [2, 8]. The viral envelope is a lipid bilayer derived from

Table 1 Molecular differences between influenza A and B viruses

RNA segment	Influenza A[1] Protein	Length (AA)	Influenza B[2] Protein	Length (AA)	Function
1	PB2	759	PB2	770	Component of RNA polymerase; Cap-binding
2	PB1	757	PB1	752	Catalytical subunit of RNA polymerase; Role RNA chain elongation
	PB1-F2*	87[3]			Pro-apoptotic activity, regulate innate immune responses, interact with PB1 to regulate polymerase activity
	PB1-N40*	718			N-terminally truncated version PB1; interacts with polymerase complex subunits, balance PB1 and PB1-F2 expression, exact function unknown
3	PA	716	PA	726	Component of RNA polymerase; Cap-snatching endonuclease subunit
	PA-X*	61[3]			Modulates host gene expression, negative virulence regulator
	PA-N155*	568			N-terminally truncated version PA; function unknown
	PA-N182*	535			N-terminally truncated version PA; function unknown
4	HA	550	HA	584	Surface glycoprotein; receptor binding and membrane fusion; antigenic determinant
5	NP	498	NP	560	Encapsidation of viral genomic RNA; RNA synthesis, nuclear import vRNA
6	NA	454	NA	486	Surface glycoprotein; neuraminidase activity, release novel virus particles after budding; antigenic determinant
			NB*	100	Ion channel activity; function unknown
7	M1§	252	M1	248	Role viral assembly, budding
	M2†	97	BM2Δ	109	Ion channel activity; essential for uncoating, role virus budding
	M42†3	99			M2 isoform with alternative ectodomain, ion channel activity; functionally complement/replace M2
8	NS1§	230	NS1§	281	Regulation viral RNA polymerase complex, interfere with antiviral state cell
	NS2/NEP†	121	NS2/ NEP†	122	Role nuclear export RNP, regulate transcription and replication
	NS3†3	174			NS1 isoform with an internal deletion; function unknown, possible role host adaptation and overcoming the species barrier

[1] On basis of A/PuertoRico/8/1934; [2] On bases of B/Lee/1940; [3] Depending on the isolate
* Alternate ORF; Δ Additional ORF, § Unspliced mRNA; † Spliced mRNA
Adapted from [2, 7], additional information from [10-13].

the host membrane and its inner surface is lined by matrix protein 1 (M1) which has an important role in viral assembly and budding [13-15]. Two major surface glycoprotein, HA and NA, are located on the outer surface of the viral envelope. HA protein is initially synthesized as a polypeptide precursor (HA0) which requires proteolytic cleavage by host cell proteases into HA1 and HA2 in order to become functionally active [16]. HA1 mainly forms the globular head region of the HA protein and encompasses the receptor-binding pocket, needed for attachment of the virus particle to the host cell [17, 18]. The HA2 represents the trans membrane (envelope) stem region of the HA protein and is essential for pH-dependent fusion of the viral envelope with the endosomal vesicle [19-21]. NA on the other hand has an important role in viral release from infected cells as it acts as a receptor-destroying enzyme that cleaves host cell sialic acid residues [22]. Matrix protein 2 (M2) is the result of alternative splicing of the matrix mRNA in case of influenza A viruses, whereas the M gene segment of influenza B viruses encompasses an additional open reading frame (ORF) directly subsequent of the M1 ORF that encodes for the BM2 protein [2, 7]. Both M2 proteins function as a transmembrane (envelope) ion channel [10, 23]. Although the M2 proteins of influenza A and B viruses are comparable in their function they differ in their respective size. Also the influenza A virus M2 channel pore is lined with hydrophobic amino acids while influenza B virus BM2 channel pores are lined with polar serines. As a result, only influenza A viruses are inhibited by the antiviral drug amantadine [10]. In addition, the M2 protein of influenza A viruses was described to have a role in virus budding [13]. A minority of the influenza A viruses express an additional ion channel protein called M42 which also results from alternative splicing of the matrix mRNA. The M42 protein represents an isoform of the M2 protein that encompasses an alternative ectodomain and was shown to functionally complement or replace the M2 protein and was demonstrated to support viral replication [24]. The expression of the highly conserved NB protein, encoded by an additional ORF in the NA gene segment, is unique for influenza B viruses. Since this protein shows ion channel activity it was long thought that the NB protein was a functional analogue of the influenza A virus M2 ion channel protein (reviewed in [11]). This theory was challenged by a study that demonstrated that NB ion channel activity was not essential for influenza B virus replication *in vitro* [25]. The true function of the NB protein remains to be established [11]. Besides the expression of structural proteins, several nonstructural proteins are expressed. Nonstructural protein 1 (NS1) is a multifunctional protein that mainly antagonizes the hosts innate immune response (further explained in the *Escape from Innate Immunity* paragraph) but is also demonstrated to have a role in regulating viral replication [26, 27]. The NS2 protein, also known as the nuclear export protein (NEP), results from alternative splicing of NS mRNA in both influenza A and B viruses and has a role in the nuclear export of RNP complexes, in regulating transcription and replication and might also aid efficient release of budding virions and host adap-

tation [28]. A limited number of influenza A viruses encode an additional splice site that gives rise to a third NS protein, namely NS3. Although its exact function remains to be established it is speculated that NS3 has a role in host adaptation and overcoming the species barrier [29]. PB1-F2 is a nonstructural protein that is expressed via an alternative ORF of the PB1 gene segment by influenza A viruses only and most likely has a role in promoting apoptosis of virus infected cells [30, 31]. Furthermore, PB1-F2 was also demonstrated to regulate innate immune responses [32, 33] and when localized in the nucleus PB1-F2 can interact with PB1 where it regulates the polymerase activity [34]. Leaky ribosomal scanning of influenza A virus PB1 mRNA results in a N-terminally truncated version of PB1, namely PB1-N40, which interacts with polymerase complex subunits like PB2. Although its exact function is currently unknown, it is speculated that it balances the expression of PB1 and PB1-F2 [35]. PA-X is also expressed via an alternate ORF of the PA gene segment by influenza A viruses only and modulates the gene expression in the host cell acting as a negative virulence regulator [36, 37]. In addition, leaky ribosomal scanning of the influenza A virus PA mRNA results in two N-terminally truncated versions of PA, namely PA-N155 and PA-N182. Although a recent study demonstrated that their expression attenuates viral replication *in vitro* and reduces the pathogenicity in mice, their exact function is still unknown [38].

Influenza virus replication cycle

Influenza virus replication is initiated by attachment of the virus HA to sialic acids on the surface of the host cell membrane (Figure 2) [18]. Human influenza A viruses normally prefer binding to α-2,6-linked sialic acids, mainly present in the human upper respiratory tract (URT), whereas avian influenza A viruses prefer α-2,3-linked sialic acids which are more abundantly present in the human lower respiratory tract (LRT) [39-42]. Influenza B viruses of the B/Victoria lineage have both α-2,3- and α-2,6-linked sialic acid binding capacities, whereas B/Yamagata viruses predominantly bind to α-2,6-linked sialic acids [43, 44]. After the initial binding, the influenza virion enters the host cell via receptor mediated endocytosis [45]. The low pH in the endosome triggers conformational changes in HA2 and thereby initiates fusion of the viral and endosomal membranes [19-21]. The M2 ion channel facilitates the influx of H+ ions into the virion, which results in uncoating of the virus particle and the release of RNPs into the cytosol (Figure 2) [10, 46, 47]. Although the exact moment of endosomal membrane fusion is unknown for influenza B viruses, it was speculated that influenza B virus RNPs might be released in the early endosome whereas influenza A virus RNPs are released in the late endosome [48]. The RNPs are then transported into the nucleus [49]. Here, the polymerase proteins drive replication of the vRNA and transcription into mRNA (Figure 2). The newly transcribed vRNAs are assembled into RNPs, which are chaperoned by M1 in order to be transported into the cytoplasm towards the apical membrane where the they

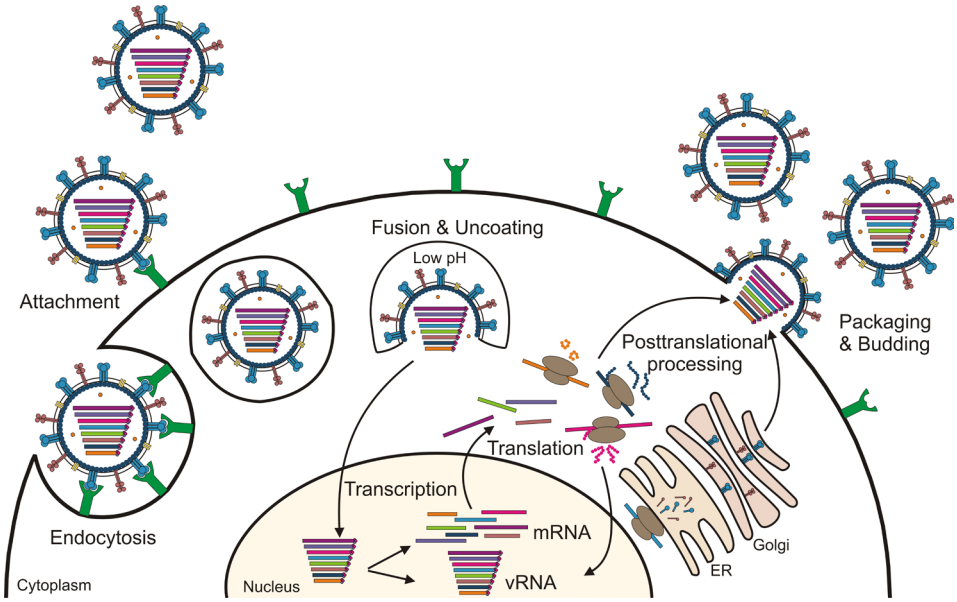


Figure 2 Influenza virus replication cycle

are packed into newly formed virus particles [47, 50]. The mRNA is also exported out of the nucleus into the cytosol where it is translated into new viral proteins. Most of these viral proteins are produced in the cytosol, although the membrane bound proteins are translated by ribosomes bound to the endoplasmic reticulum (ER) membrane [51]. These are subsequently transported via the golgi apparatus, where post translational modifications are made, to the cells' cytosolic membrane where novel virus particles are assembled (Figure 2) [52]. Here, the M1 protein has accumulated under the host cell membrane and serves an important role in viral assembly as it interacts with both the viral envelope and the RNP complexes. M1 also initiates the membrane to bud outwards until the cell membrane fuses at the bottom of the novel virion to close it (Figure 2) [13-15]. Enzymatic activity of the NA mediates the actual release of the virions from the cell as it prevents binding of the newly formed virus particles to the host cell by cleaving the sialic acids on the cell surface [22].

Natural hosts of influenza viruses

Influenza A viruses

Influenza A viruses have a large animal reservoir. Aquatic birds are the natural reservoir for all influenza A subtypes except for the recently discovered H17N10 and H18N11 viruses which have only be detected in bats [3-5, 53, 54]. Of interest serological evidence indicates that bats may also harbor other influenza A virus subtypes [55]. Avian influenza A viruses are occasionally transmitted to mammals.

Influenza A virus infections have been observed in pigs (*Sus domesticus*), horses (*Equus ferus caballus*), dogs (*Canis lupus familiaris*), marine mammals (seals and whales), domestic cats (*Felis catus*), tigers (*Panthera tigris*), leopards (*Panthera pardus*) and many more [56-60]. The H5N1 subtype alone has been shown to naturally infect over 150 species including a large variety of birds [61]. Avian influenza viruses occasionally cross the species barrier into the human population (see also *Influenza viruses epidemics and pandemics* paragraph).

Influenza B viruses

In contrast to influenza A viruses, there is no clear evidence that influenza B viruses continuously circulate among animals. However, influenza B viruses were isolated from dogs, harbor seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*), indicating that influenza B viruses are able to infect these species [62, 63]. In addition, there is serological evidence for infection of influenza B viruses of various animal species that live in close proximity to humans, including dogs, guinea pigs (*Cavia porcellus*), pigs, horses, ruminants, Bornean orangutans (*Pongo pygmaeus*), Western common chimpanzees (*Pan troglodytes verus*), Western lowland gorillas (*Gorilla gorilla gorilla*) and zoo birds [64]. Of interest, influenza B virus-specific antibodies have also been detected in various species of wild pinnipeds; Caspian seals (*Phoca caspica*), grey seals (*Halichoerus grypus*), harbor seals (*Phoca vitulina*), South American fur seals (*Arctocephalus australis*), but not in harbor porpoises (*Phocoena phocoena*) [64]. The results of these studies indicate that influenza B viruses are able to occasionally infect various species of animals, but there is no epidemiological evidence for a role of these species as reservoir of influenza B viruses that could spill over to humans, like influenza A viruses in wild birds.

Influenza viruses epidemics and pandemics

Seasonal epidemics

Influenza viruses infections are one of the leading causes of respiratory tract infections in humans [2], causing an estimated 3-5 million severe clinical infections and resulting in 250,000-500,000 fatal cases annually [65, 66]. Seasonal influenza epidemics are caused by influenza A viruses of the H3N2 (A/H3N2) and H1N1 (A/H1N1) subtype, and influenza B viruses [65]. Annual recurrence of these viruses is attributed to their ability to evade recognition by virus-specific neutralizing antibodies induced after previous infections or vaccinations by accumulating mutations mainly in the HA glycoprotein, a process known as antigenic drift (Figure 3) (see also the *Escaping the humoral immune response* paragraph) [67-70]. Clinical signs after infection include nasal obstruction, cough, fever, sore throat, myalgia and headache but complications like severe pneumonia do occur, especially among children younger than 2 years of age, adults over 65 years of age and the immunocompromised [65]. Early beliefs were that influenza B viruses cause less

severe disease than influenza A viruses, although hospitalization rates, morbidity or mortality (excluding influenza A virus pandemics) did not support this [71]. Influenza B virus infections are now generally considered less severe than those caused by A/H3N2 viruses, but more severe than A/H1N1 infections [72-78].

On the other hand, influenza viruses of an antigenically distinct (novel) subtype can be introduced into the human population a process better known as antigenic shift (Figure 3). When such an antigenically-distinct virus is transmitted efficiently from human-to-human, it may cause a pandemic influenza outbreak, since neutralizing antibodies to this virus are absent in the population at large [79]. In the last century four major pandemic outbreaks have emerged, of which at least three were the result of genetic reassortment between animal and human influenza A viruses (Figure 4) [80-87].

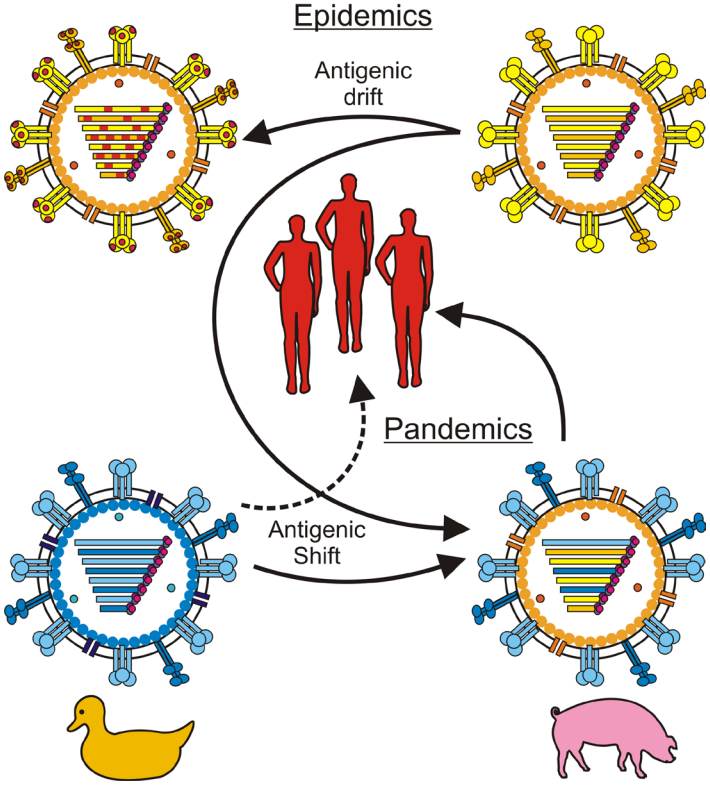


Figure 3 Antigenic drift and shift to escape immunity

The gradual accumulation of mutations, mainly in the highly variable globular head region of HA, causes the influenza A and B viruses to escape recognition by virus neutralizing antibodies and allows it to cause seasonal epidemic outbreaks. This phenomenon is called antigenic drift. The introduction of a novel influenza A virus subtype into the human population is called antigenic shift and may cause a pandemic outbreak in the naïve human population when the virus is efficiently transmitted from human-to-human, since antibodies directed against the novel subtype are absent. Past pandemic outbreaks were caused by exchange (reassortment) of gene segments between two or more influenza strains, e.g., avian and human. However, studies in ferrets suggest that avian influenza viruses, like H5N1, could be directly transmitted from animal reservoirs into the human population, requiring only a small number of adaptive mutations [88] as indicated by the dotted line in this figure.

Spanish A/H1N1 pandemic (1918)

In 1918, in a matter of months, an influenza A virus of the H1N1 subtype spread worldwide, killing 25-50 million people [80, 89] with an unusual high attack rate among previously healthy young adults [90, 91]. This outbreak became known as the "Spanish Flu". For long it was speculated that the 1918 A/H1N1 pandemic resulted from an avian A/H1N1 virus that had directly crossed the species barrier into the human population [92]. However, more recent studies suggest that this virus resulted from multiple reassortment events between avian, swine and human influenza viruses [81, 82, 93]. Following the initial pandemic the A/H1N1 virus continued circulating in the human population, causing seasonal epidemics for almost four decades (Figure 4).

Asian A/H2N2 pandemic (1957)

The A/H1N1 virus was eventually replaced in 1957, when a novel influenza A virus of the H2N2 subtype emerged. The "Asian Flu" pandemic was responsible for an estimated 2-3 million deaths worldwide [94]. The A/H2N2 virus was the result of a reassortment event that gave rise to a virus that had obtained the PB1, HA and NA gene segments from an avian A/H2N2 virus and the remaining gene segments originated from the human A/H1N1 virus that circulated prior to 1957 (Figure 4) [83, 95, 96]. This virus continued to circulated epidemically for another 11 years.

Hong Kong A/H3N2 pandemic (1968)

In 1968, the A/H2N2 virus was replaced by an influenza A virus of the H3N2 subtype, which caused the "Hong Kong Flu" pandemic [97]. The A/H3N2 virus resulted from a reassortment event between an avian H3N? virus and the previously circulating human A/H2N2 virus, the PB1 and HA gene segments were obtained from the avian influenza virus (Figure 4) [83, 95, 98]. This pandemic was considerably milder as the previous two pandemics with an estimate death toll of 1 million worldwide [94]. The A/H3N2 has seasonally circulated in the human population ever since.

Re-emergence of A/H1N1 (1977)

In 1977, an A/H1N1 virus started to circulate in the human population which was genetically very similar to viruses that had circulated in the early 1950s, which makes it plausible that this virus was accidentally released from a laboratory or live-attenuated vaccine trail (Figure 4) [99-101]. Fortunately this did not lead to another pandemic outbreak. This A/H1N1 virus seasonally co-circulated with the A/H3N2 virus until 2009.

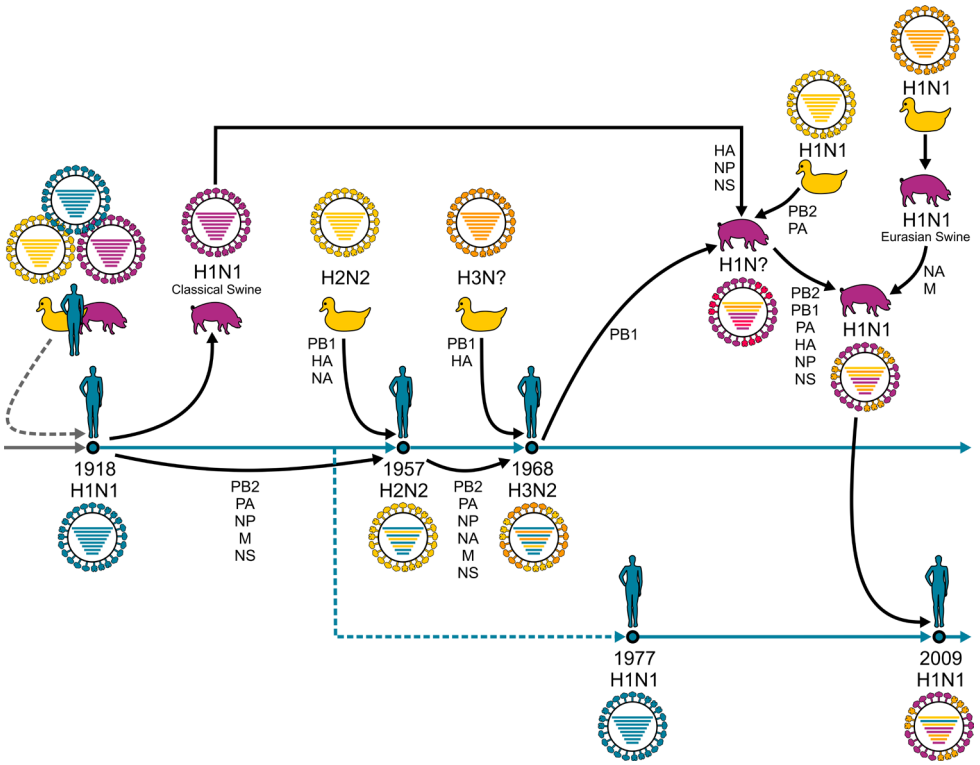


Figure 4 Reassortment events of pandemic influenza A viruses
The 1918 A/H1N1 virus possibly originated from multiple reassortment events between avian, swine and human viruses. This A/H1N1 virus continued to circulate, causing seasonal epidemics, until 1957 when a novel A/H2N2 virus emerged after a reassortment event with an avian A/H2N2 virus. This virus circulated until 1968, when it reassorted with an avian A/H3N? virus. The novel A/H3N2 virus caused seasonal epidemics ever since. A/H1N1 was reintroduced in the human population in 1977 and co-circulated with A/H3N2 viruses until 2009 when it was replaced by H1N1pdm09, which originated after multiple reassortment events between avian, swine and human viruses.

Swine A/H1N1 pandemic (2009)

The first pandemic outbreak of the 21th century occurred in 2009, when an novel influenza A virus of the H1N1 subtype (H1N1pdm09) emerged in Mexico and replaced the previously circulating A/H1N1 virus. This virus was the result of multiple reassortment events between avian (H1N1; PB2, PA), classical swine (H1N1; HA, NP and NS), Eurasian swine (H1N1; NA and M) and human (H3N2; PB1) influenza A viruses (Figure 4) [84, 86, 87]. The pandemic was considered relatively mild in terms of morbidity and mortality, with an estimated 0,15 – 0,57 million deaths worldwide [102]. The H1N1pdm09 virus preferentially affected young adults, whereas people over 65 years of age generally experienced a relatively mild infection. This was partially contributed to the presence of antibodies in the elderly population which cross-reacted with the 2009 H1N1pdm09 virus, as it was antigenically similar to a H1N1 virus that circulated prior to 1957 [103]. As from 2009, the H1N1pdm09 co-circulates with the A/H3N2 subtype in the human population.

Pandemic threats

It was estimated that if nowadays a pandemic influenza virus were to emerge with a similar severity as the 1918 A/H1N1 strain global mortality would rise to 51-81 million [89]. Therefore influenza viruses that cross the species barrier into the human population are closely monitored [104]. Avian influenza A viruses of various subtypes, including H5N1, H5N6, H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7 and H10N8, have occasionally infected humans during the last two decades [105, 106]. Also, viruses of swine origin, like a swine reassorted variant of the H3N2 virus (H3N2v), has been reported to occasionally infect humans [107]. Fortunately, most zoonotic transmissions occur without sustained human-to-human transmission.

In 1999 two isolated human cases of influenza A/H9N2 virus infections were reported in Hong Kong, displaying mild symptoms only [108, 109]. A study by Sorrel *et al* demonstrated that influenza A viruses of the H9N2 subtype could result in airborne transmission between ferrets, whose respiratory tract closely resembles that of humans, if the A/H9N2 virus were to reassort with a human A/H3N2 virus [110]. Studies have demonstrated that pigs (and quails) could serve as a mixing vessel for emerging reassorted influenza A viruses [85, 111, 112]. As both avian A/H9N2 and human A/H3N2 viruses were found to circulate in pigs, such a reassortment event might occur resulting in the emergence of an A/H9N2 variant capable of airborne transmission between humans [113-115].

Larger is the impact of highly pathogenic avian influenza A/H5N1 viruses which have been transmitted on a regular basis from infected poultry to man since the first case was identified in 1997 in Hong Kong [116]. Since 2003, over 844 human cases have been reported from 16 countries, most of them suffering from severe pneumonia progressing to acute respiratory distress syndrome (ARDS), resulting in a case fatality of 50% [104, 117-120]. The reported case fatality rate most likely is an overestimate, since subclinical infections and mild cases are not reported [121]. So far, sustained human-to-human transmission has not been observed, although clusters of human cases have been reported [122-124]. Furthermore, recent studies have shown that, in principle, transmission of highly pathogenic A/H5N1 viruses amongst mammals is possible and that only a limited number of adaptive mutations are required for airborne transmission, emphasizing the pandemic potential of these viruses [88, 125, 126].

During an outbreak of highly pathogenic avian influenza A virus of the H7N7 subtype in the Netherlands in 2003, 89 human cases were reported of which one had a fatal outcome [127, 128]. A zoonotic transmission of A/H7N7 virus subtype was previously reported in the United Kingdom in 1996 [129]. Other avian viruses of the H7 subtype have been reported to sporadically infect humans, including viruses of the H7N2 [130-132] and H7N3 subtypes [133-136].

An avian influenza A virus of the H7N9 subtype that has emerged recently poses a more serious pandemic threat. Over 677 human laboratory-confirmed cases, of

which 275 had a fatal outcome, have been reported since the first human case was reported in China in February 2013 [104]. Most hospitalized patients develop severe viral pneumonia and ARDS [137-140]. A/H7N9 virus infections are more frequently observed in the winter months, and seem to have a similar seasonality as the seasonal human influenza viruses [141]. The A/H7N9 virus most likely resulted from multiple reassortment events between three avian viruses [138, 142, 143]. The pandemic potential of this A/H7N9 virus is further underlined by the presence of the Q226L substitution in the HA [138, 144]. This mutation is associated with binding to α-2,6-linked sialic acids found in the human URT [145] and with airborne transmission of the avian A/H5N1 virus in ferrets [88]. In case of the A/H7N9 virus only limited airborne transmission has been observed between ferrets [145-149]. Although a number of human A/H7N9 clusters were reported, this has not led to sustained human-to-human transmission of the A/H7N9 virus so far [150-153]. The report of an A/H7N9 patient who was co-infected with a seasonal human A/H3N2 virus underscores the rational that the A/H7N9 virus might further adapt to humans by acquisition of gene segments from human seasonal influenza A viruses through genetic reassortment [81, 83, 84, 86, 87, 154, 155].

Influenza B viruses are epidemic but not pandemic

The earliest influenza B virus isolate originates from the 1940s, well before the emergence of two antigenically distinct influenza B lineages [67, 156, 157]. Currently, two influenza B lineages are distinguished on basis of their HA glycoprotein; the B/Victoria lineage (named after the B/Victoria/2/1987 strain) and the B/Yamagata lineage (named after the B/Yamagata/16/1988 strain) [6, 67, 70, 158]. It is thought that the two lineages diverged in the 1970s, when the B/Victoria lineage gradually emerged in China [157, 158]. The insulated state of China in the 1970s may have delayed the global spread of the B/Victoria lineage. Consequently, B/Yamagata was the major circulating lineage until B/Victoria made a global appearance in the mid-1980s [158]. Viruses of both influenza B lineages co-circulate with seasonal A/H3N2 and A/H1N1 viruses ever since [65]. The incidence of infections with influenza B viruses can vary drastically between influenza seasons [159]. Between 1985 and 2000 the influenza seasons were always dominated by a single influenza B virus lineage, B/Victoria in the late 80s and B/Yamagata in the 90s, but since 2001 both lineages have been co-circulating in most influenza seasons both in the northern and southern hemisphere [70, 159, 160]. However, there is no conclusive evidence for continuous circulation of influenza B viruses in animal species. Consequently, this limits the potential for zoonotic transmission of antigenically distinct influenza B viruses and thereby the risk of pandemic outbreaks.

Immunity to influenza viruses

Without lifelong protection against seasonal influenza virus infections and the threat of possible future pandemics, it is of great importance to have insight in how immunity against influenza infections is formed and how influenza viruses manage to evade these immune responses. In contrast to immunity to influenza A viruses, immune responses to influenza B viruses are poorly studied. Hence, most of our knowledge about immunity to influenza viruses is bases on studies with influenza A viruses.

Innate immunity

The primary targets for influenza viruses are the epithelial cells that line the respiratory tract and which initiate an antiviral immune response upon detection of the virus. The first line of defense is formed by the innate immune system, which is quick but lacks specificity and memory. Innate immunity is formed by physical barriers (e.g., mucus and collectins) and innate cellular immune responses [7, 161].

Sensing of influenza virus infection by receptors of the innate immune system

Influenza virus infection results in the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that initiate antiviral signaling cascades, resulting in the production of interferons (IFNs), cytokines and chemokines [162]. Three main categories of PRRs are involved in recognition of an influenza infection and the induction of an IFN response: Toll like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) receptors and nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) [163-165].

The TLRs are the first receptors to recognize the influenza virus infection. TLR7 is an intracellular receptor that recognizes single stranded viral RNA (ssRNA) after the ribonucleoprotein complex has been degraded inside acidified endosomes [166, 167]. TLR3 is another intracellular receptor that recognizes double stranded viral RNA (dsRNA) [168]. Other TLR receptors likely to sense an influenza virus infection are TLR2 and TLR4, which are present on the cell surface and recognize viral surface glycoproteins like influenza HA and NA [165, 169-171]. At a later stage of infection, newly produced uncapped, 5'-triphosphates bearing viral RNAs are recognized by RIG-I receptors in the cytoplasm [172-175]. NLRP3 is part of the NLRP3 inflammasome and is activated by dsRNA which subsequently activates caspase I, resulting in the proteolytic maturation of IL-1β and IL-18 [176].

The signaling cascade of all activated TLRs, except for TLR3, starts with the activation of MyD88, which subsequently can activate tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6), either directly or via IL-1R-associated kinase-1 (IRAK 1), eventually leading to the activation of mitogen-activated kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). TLR3 signaling cascade starts with the activation of TRIF (TIR-domain-containing adapter inducing interferon-β)

eventually activating NF-κB and interferon regulatory factor 3 (IRF3). TLR4 can also signal via the TRIF dependent pathway by formation of a TRAM-TRIF complex.
Activation of RIG-I receptor by binding of viral 5'-triphosphates RNA or viral dsRNA to the repressor domain (RD) of RIG-I results in conformational changes exposing the caspase activation and recruitment domains (CARDs). These domains are ubiquitinated by IFN-inducible E3 ubiquitin ligase, tripartite motif 25 (TRIM25) [177]. RIG-I can then associate with mitochondrial antiviral signaling adaptor (MAVS; also known as IPS-1, VISA or Cardif), starting a signaling cascade that leads to the activation of IRF3 and NF-κB. The signaling cascades via TLRs and RIG-I receptors have been extensively studied (Figure 5) [26, 175, 178, 179].
All these pathways eventually result in the transcription of proinflammatory cytokines, chemokines and IFNs that activate the antiviral response and the recruitment of neutrophils, activation of macrophages and maturation of dendritic cells (DCs) [162]. So far, three IFN types have been identified [180]. Type I IFNs include IFN-α and IFN-β which have an important role in limiting viral replication [181, 182]. Type I interferons secreted by infected cells act on IFN-α/β receptors (IFN-α/βR) of the same cell or neighboring cells, activating an antiviral signaling cascade that involves phosphorylation of tyrosine kinase 2 (Tyk2) and Janus kinase 1 (Jak1), also called, "just another kinase 1", which then phosphorylate signal transducer and activators of transcription (STAT) 1 and STAT2. Phosphorylated STAT1 and 2 combine with IRF9 to form ISGF3 (IFN-stimulated gene factor-3 transcription factor complex) which is responsible for the transcription of >300 genes that encode for e.g., antiviral proteins (Table 2) that establish an antiviral state in the cell that limits viral replication (Figure 6) [179]. IFN-β acts through a positive feedback loop on the IFN-β receptor which activates IFN stimulated gene factor 3 (ISGF3), resulting in the expression of IRF-7. IRF-7 is phosphorylated in the presence of a viral infection and induces the expression of both IFN-α and IFN-β [183]. IFN-γ is the main type II IFN and contributes to the establishment of an effective adaptive cytotoxic T cell (CTL) response against the influenza virus infection. It regulates virus-specific CTL homeostasis in secondary lymph nodes and subsequent trafficking of CTLs to the site of infection [196]. Furthermore, IFN-γ plays an important role in memory CTL responses [197]. Type III IFNs, like IFN-λ, also control influenza A infections in the lung [198].

Table 2 IFN-induced antiviral proteins and their function

Protein	Function	Reference
MxA (Myxovirus resistance gene A)	Inhibits viral replication by interfering with the viral ribonucleoprotein structure	[184-186]
PKR (Protein kinase R)	Limits viral replication by blocking general translation	[187, 188]
OAS (2'–5'oligoadenylate synthetase)	Stops viral replication by means of activating RNAseL which results in degradation of viral and cellular RNA and eventually apoptosis of the virus infected cell	[189, 190]
ISG15 (IFN-stimulated gene 15)	Regulates a number of IFN-stimulated proteins	[191]
Viperin	Inhibits viral release by interfering with viral budding	[192]
Tetherin	Inhibits formation of influenza virus particles	[193, 194]
IFITMs	Restrict viral entry	[195]

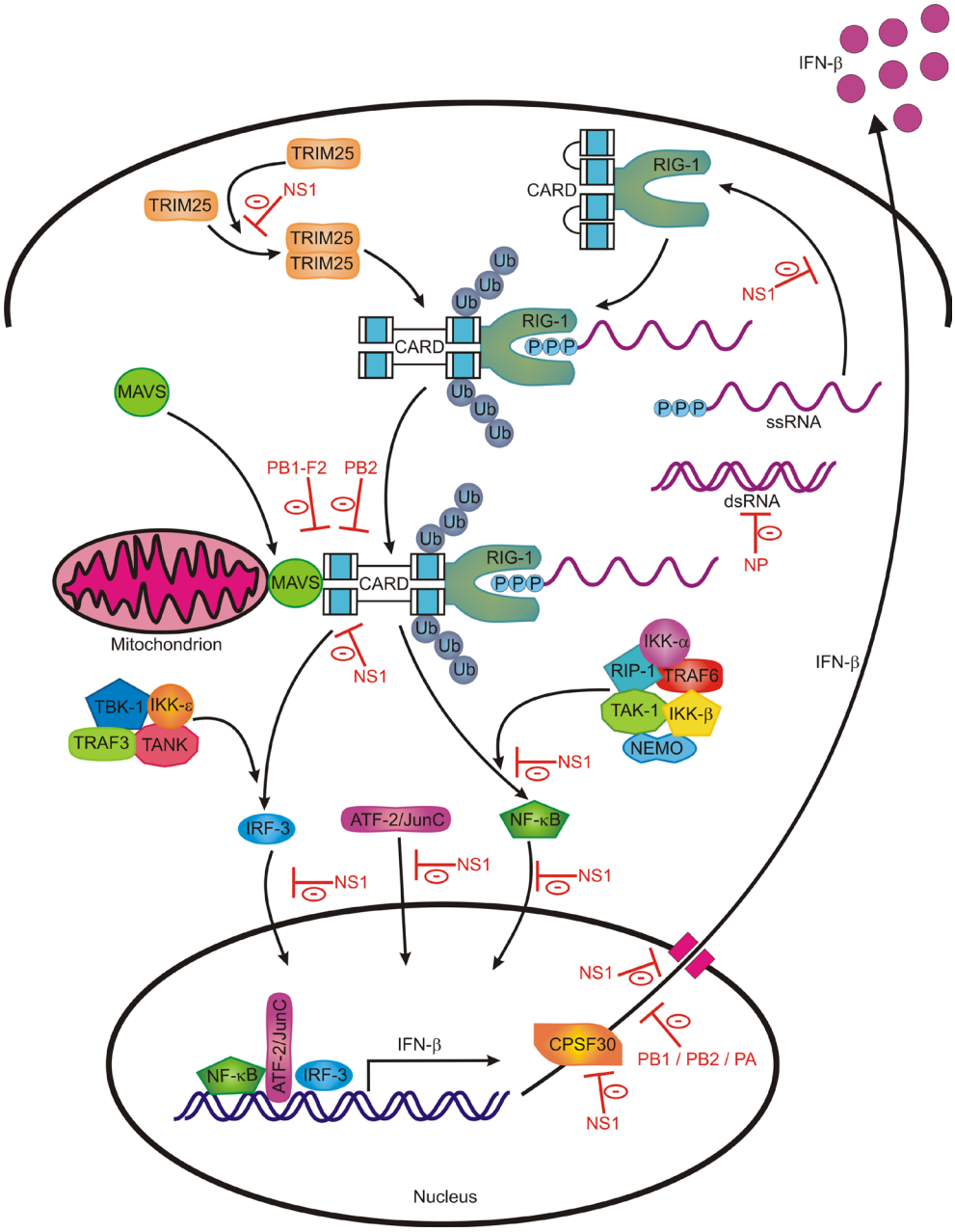


Figure 5 The RIG-I signaling pathway and inhibition by influenza A viruses
By-products of viral replication are 5'-triphosphates ssRNA and dsRNA which can bind to the RIG-1 receptor, leading to conformational changes, causing exposure of the CARDS which are ubiqutinated by TRIM25. Subsequently, RIG-1 associates with MAVS and thereby starts a signaling cascade leading to activation of transcription factors IRF3, NF-κB and ATF-2/JunC, resulting in the transcription of IFN-β mRNA. Indicated in red are sites at which the influenza A virus interferes with this pathway, as explained in the text. (Figure adapted from [26]).

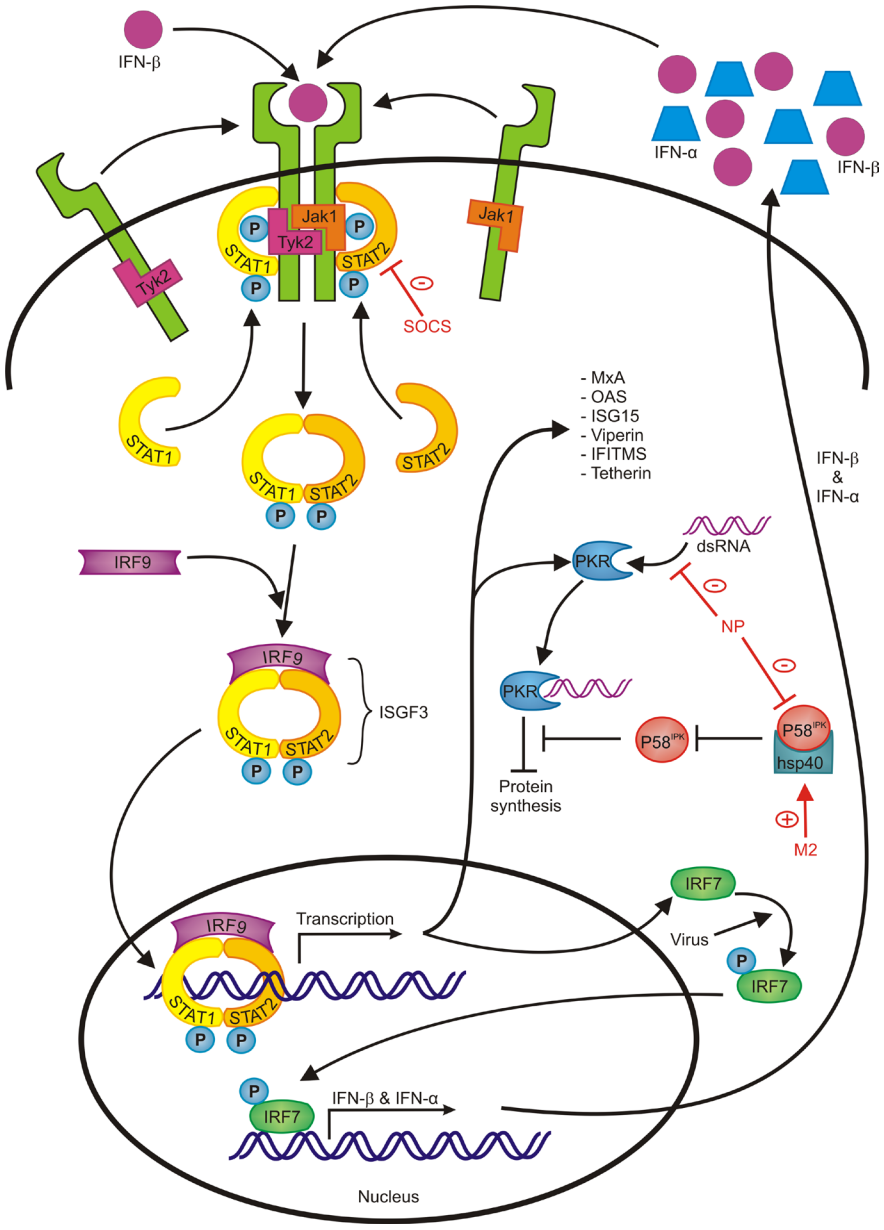


Figure 6 The type I IFN signaling pathway and inhibition by influenza A viruses
IFN-β produced by influenza virus-infected cells binds IFN receptors causing the phosphorylation of Tyk2 and Jak1. This is followed by binding and phosphorylation of STAT1 and STAT2 which subsequently form a complex with IRF9. This ISGF-3 complex acts as a transcription factor for >300 genes, several of which display an antiviral effect (see text). The expressed protein PKR is activated upon recognition of viral dsRNA, leading to inhibition of protein synthesis, including viral proteins. PKR is inhibited by the cellular protein $P58^{IPK}$, however $P58^{IPK}$ activity is downregulated by binding cellular hsp40. The IRF7 protein is phosphorylated in the presence of influenza A virus, leading to activation of a positive feedback loop, causing the transcription of IFN-α and IFN-β. Indicated in red are mechanisms of the influenza A virus to interfere with this pathway, these interfering mechanisms are explained more extensively in the text. (Figure adapted from [26]).

Macrophages

During homeostasis, alveolar macrophages exhibit a relatively quiescent state, producing only low levels of cytokines, and suppress the induction of innate and adaptive immunity [199, 200]. CCL2, produced by infected epithelial cells during the initial phase of the influenza virus infection, attracts alveolar macrophages and monocytes via their CCR2 receptor [201-203]. Activated macrophages enhance their pro-inflammatory cytokine response, including IL-6 and TNF-α [204, 205]. Alveolar macrophages have a direct role in limiting viral spread by phagocytosis of apoptotic infected cells [171, 206, 207] and by phagocyte-mediated opsonophagocytosis of influenza virus particles [208]. They are also involved in regulating the adaptive immune response. Depletion of alveolar macrophages prior to influenza virus infection led to a reduction of antibody titers and reduced numbers of virus-specific CTLs post-infection [207]. In contrast to these beneficial effects, alveolar macrophages also pose a negative effect, since their activation also results in the production of nitric oxide synthase 2 (NOS2) and TNF-α which contribute to the severe pathology that can be the result of an influenza virus infection [201, 209, 210].

Natural Killer cells

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system. They are able to lyse infected cells in a MHC class I independent manner via a direct or indirect mechanism of recognition. The sialylated NKp44 and NKp46 receptors are bound by the HA proteins expressed on the surface of influenza virus-infected cells [211]. This results in direct lysis of the infected cell [212, 213]. It was shown that mice lacking the NKp46 receptor equivalent, NCR-1, displayed increased morbidity and mortality following influenza A infection [214]. NK cells with their CD16 receptor (FcγRIII) can bind to the Fc portion of antibodies bound to influenza virus-infected cells and mediate lysis of these cells. This process is known as antibody-dependent cell cytotoxicity (ADCC) [215-217].

Dendritic Cells

Dendritic cells (DCs) are professional antigen presenting cells (APCs) which form an important bridge between the innate and the adaptive immune system. During an infection, DCs initiate adaptive immune responses by the presentation of viral antigens to naïve and memory T and B lymphocytes (Figure 7) [199, 218, 219]. At steady state conditions, DCs constantly survey the lungs for invading pathogens or foreign material [220]. Once the lungs are infected with an influenza virus, the DCs can acquire the antigens via two distinct mechanisms. The first route is by direct infection of DCs by the influenza virus [221, 222]. Proteasomes in the cytosol degrade viral proteins into small peptides which are transported to the endoplasmic reticulum (ER) via TAP (transporter of antigen processing), where they are loaded to MHC class I molecules. These MHC class I peptide complexes are then transported

via the Golgi complex onto the cell membrane where they can be recognized by influenza virus-specific CD8⁺ cytotoxic T cells (CTLs) (Figure 8) [218, 223]. The second mechanism of antigen acquisition by DCs is through phagocytosis of virus particles or apoptotic epithelial cells [218, 224-226]. Viral proteins are then degraded into smaller peptides in endosomes/lysosomes and presented on the cell surface in MHC class II peptide complexes which can be recognized by CD4⁺ T helper cells. T helper cells assist B cells to proliferate and mature into antibody-producing plasma cells. Via this route of antigen acquisition, DCs can also present epitopes to CD8⁺ T cells. This is also known as cross-presentation. For presentation of viral antigens to virus-specific T cells, activated DCs migrate to the draining lymph nodes [220, 227-229].

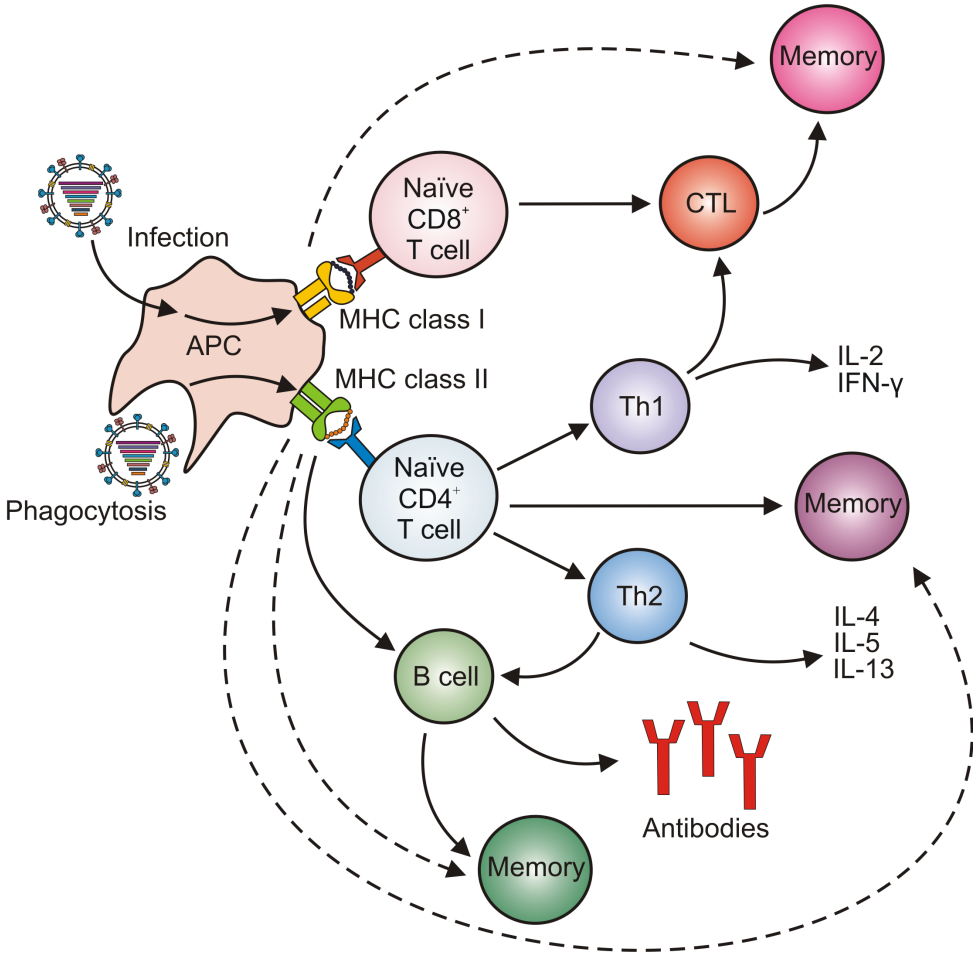


Figure 7 Induction of humoral and cellular immunity
Induction of immune responses after a primary influenza virus infection is indicated by solid arrows. The more rapid activation of virus-specific memory cell populations upon secondary encounter with an influenza A virus are indicated by dotted arrows.

Adaptive immunity

The second line of defense against influenza virus infection is the adaptive immune response. Overall, this highly specific response is relatively slow upon first encounter with a pathogen. However, as a result of the formation of immunological memory, the response is faster and stronger after a second encounter with the same pathogen. The adaptive immune response consists of humoral (virus-specific antibodies) and cellular (virus-specific $CD4^+$ and $CD8^+$ T cells) immunity.

Humoral immunity

Influenza virus infection induces the production of influenza virus-specific antibodies by B cells [230-233]. In particular, antibodies directed to the viral HA and NA correlate with protective immunity.

Antibodies directed to the trimeric globular head of HA can afford sterilizing immunity to influenza virus infection. By binding to the HA receptor binding site located in this region they can block virus attachment to host cells and/or block receptor-mediated endocytosis [234-236]. However, most antibodies directed against HA are influenza virus strain-specific and fail to neutralize intrasubtypic drift variants and viruses of other subtypes [79, 237-240]. This is mainly due to the high variability in the HA globular head (see also the *Escaping the Humoral Immune Response* paragraph). Of interest, humoral immunity elicited after an influenza virus infection does provide long-lasting antibody mediated protection against strains that resemble the infected strain. This was exemplified during the pandemic of 2009 caused by an influenza A virus of the H1N1 subtype. Elderly people that were exposed to influenza A/H1N1 virus in the 1950s had antibodies which cross-reacted with the pandemic strain and were relatively spared from contracting infections and developing disease [103, 240-242]. Recently, broadly reacting antibodies directed against the conserved stem region of HA have been identified for influenza A [243-251] and B viruses [252, 253].

Antibodies to the other major glycoprotein, the viral NA, interfere with the last phase of the viral replication cycle and also exert protective immunity. Unlike HA-specific antibodies, NA-specific antibodies do not neutralize the virus. However, by inhibiting the enzymatic activity of NA, these antibodies limit the viral spread and thus shorten severity and duration of illness [254-258]. Furthermore, NA-specific antibodies may also contribute to clearance of virus-infected cells by facilitating ADCC [215].

In addition to HA and NA, influenza virus particles contain the minor glycoprotein M2. A protective effect of M2-specific antibodies was first demonstrated in mice after passive transfer of M2-specific monoclonal antibodies [259, 260]. M2-specific antibodies facilitate ADCC [217, 261, 262]. Since the M2 protein is highly conserved between various influenza A virus subtypes and the two influenza B lineages, M2-specific antibodies are likely to afford heterosubtypic immunity [263-270].

After infection, antibodies are also induced against other viral proteins, including NP [271]. Since NP is highly conserved between influenza A or B viruses, these antibodies could potentially contribute to heterosubtypic immunity. Although NP-specific antibodies are non-neutralizing, it was shown in mice that they contribute to protective immunity [272, 273]. However, their mode of action is poorly understood, but may include ADCC of infected cells and opsonisation of NP, resulting in improved T cell responses [274, 275].
After primary infection with influenza virus, serum antibodies of the IgM, IgA and IgG isotypes are induced, whereas after secondary responses, IgM responses are not observed [276]. IgM antibodies can neutralize the virus, but also activate the complement system [277, 278]. In humans, virus-specific serum IgA responses seem indicative for recent infection with influenza virus [279, 280]. Virus specific IgG antibodies correlate with long-lived protection, provided that the antibodies match the strains causing the infection [281-283]. In addition to serum antibodies, influenza virus infection also induces local mucosal sIgA antibody responses that protect airway epithelial cells from infection [282, 284, 285].
Young infants may be protected from influenza virus infection by maternal antibodies, when they match the incoming virus [286-290].

Cellular immunity

Influenza virus infection induces a cellular immune response, including virus-specific $CD4^+$ T cells and $CD8^+$ T cells. These cells play an important role in regulation of the immune response and viral clearance respectively.

$CD4^+$ T cells

$CD4^+$ T cells are activated after recognition of viral epitopes associated with MHC class II molecules and interaction with co-stimulatory molecules on APCs. Depending on the cytokine milieu, activation of naïve $CD4^+$ T cells can result in the differentiation into $CD4^+$ T helper 1 (Th1) or Th2 cells, which can be distinguished based on their cytokine expression profiles [291]. Th2 cells produce IL-4, IL-5 and IL-13 and promote the activation and differentiation of B cells, resulting in antibody production [292-294]. The antibody response is strengthened by the induction of antibody class switching and somatic hypermutations affecting the variable region of the antibody, resulting in affinity maturation of the influenza-specific antibodies [295-298]. Th1 cells produce IFN-γ and IL-2 and are mainly involved in promoting CTL responses [299, 300], and are essential for the induction of memory $CD8^+$ T cells [301-303]. Memory $CD4^+$ T cells, induced after a primary influenza A virus infection, contribute to faster control of subsequent influenza A virus infections [304]. Lung-resident memory $CD4^+$ T cells in particular have an important role in protection against secondary influenza A virus infections [305]. In addition to helper function, $CD4^+$ T cells also display cytolytic activity [306, 307]. It was

shown that these cells play a role in protective immunity against influenza A virus infections in humans [308], including in heterosubtypic immunity [309]. The role of $CD4^+$ T cells in influenza B virus infections is less well studied.

$CD8^+$ T cells

Naïve $CD8^+$ T cells are activated after recognition of viral epitopes associated with MHC class I molecules on APCs in the draining lymph nodes, and subsequently differentiate into CTLs. These CTLs migrate to the site of infection where they recognize and eliminate influenza virus infected cells and thereby prevent the production and spread of progeny virus [310]. Human influenza virus-specific CTLs are mainly directed against epitopes of the highly conserved internal viral proteins, like M1, NP, PA and PB2. Therefore, CTLs display a high degree of cross-reactivity with influenza A viruses of various subtypes [311-317]. T cell receptor (TCR) activation by a specific epitope-MHC class I complex results in a lytic response, mediated by the release of perforin and granzymes causing apoptosis of the infected cell [318-320]. Furthermore, proinflammatory cytokines are produced, like TNF-α, which also inhibit virus replication and enhance lytic activity [318, 321-324]. Also, FasL expression is upregulated which promotes apoptosis of infected cells [320]. After infection, a pool of long-lived antigen-specific central memory and effector memory $CD8^+$ T cells is formed, which form the basis for more rapid and stronger recall responses upon secondary infections [325-334].

Much of the current knowledge about the protective role of $CD8^+$ T cells in influenza A virus infections has been obtained from mouse studies which showed that $CD8^+$ T cells contribute to homo- and heterosubtypic immunity [335-342]. Evidence that CTLs protect against influenza in humans is sparse. A recent study indicated the presence of heterosubtypic memory $CD4^+$ and $CD8^+$ T cells against the 2009 pandemic A/H1N1 virus in naïve individuals [343]. The presence of these cells correlated with protection against severe disease caused by a 2009 pandemic A/H1N1 virus infection [344]. It was shown that the extent of lytic activity of PBMC inversely correlated with the extent of virus shedding after experimental infection of subjects that lacked antibodies to the live attenuated strain used for infection [345]. More circumstantial evidence for a protective role of $CD8^+$ T cells in heterosubtypic influenza infections in humans comes from epidemiological studies. People who had a symptomatic influenza A infection with the A/H1N1 strain prior to the 1957 pandemic were partially protected from infection with the pandemic A/H2N2 strain [346, 347]. A similar trend was found in isolated infections with the A/H5N1 [348]. Little is known about the role of $CD8^+$ T cells in influenza B virus infections. It remains to be established whether influenza B virus-specific CTLs are able to cross-react between viruses of the two influenza B lineages.

Regulatory T cells and Th17 cells

In addition to the activation of virus-specific $CD4^+$ T cells and $CD8^+$ T cells, two other cell types are activated, namely forkhead box P3 $(FOXP3)^+$ regulatory T cells (T_{regs}) and T helper 17 (Th17) cells. T_{regs} play an important role in balancing the immune response during infection. They control $CD4^+$ T helper cell and CTL responses in order to prevent immunopathology of infected tissues [324, 349, 350]. Th17 cells produce IL-6 which inhibits the effects of T_{regs} and therefore promote T helper responses [349]. Furthermore, Th17 cells have a role during influenza infections in counteracting secondary bacterial infections, e.g. *S. aureus* pneumonia. Influenza A virus infections may promote secondary bacterial pneumonia by suppressing Th17 cells in an type I IFN-dependent manner [351].

Evasion of the antiviral immune response by influenza viruses

Immune pressure on influenza viruses forces them to adopt strategies to evade immunity. Binding of influenza viral proteins to various components of the innate immune system leads to their inhibition (Figures 5 and 6) [26], whereas a combination of immune pressure in the human population and the high mutation rate of influenza viruses leads to the generation of new virus strains that escape the existing adaptive humoral and cellular immune responses (Figures 3 and 8). As immune responses to influenza B viruses are less well studied than immune responses against influenza A viruses, most of our knowledge about viral evasion strategies described here is based on studies that used influenza A viruses. Individual evasion strategies adopted by influenza B viruses are indicted.

Escape from innate immunity

Upon infection, innate antiviral immune response are first induced. Viral RNA of both influenza A and B viruses are encapsidated by NP to reduce the formation of dsRNA which could otherwise lead to activation of RIG-I signaling. Since most PRRs are located inside the cytoplasm, the nuclear replication strategy of the influenza viruses prevents the recognition of viral RNA by cytosolic PRR. However, the innate IFN response is initiated much faster upon an influenza B virus infection than after influenza A virus infection [352]. Although the exact moment of endosomal membrane fusion and the subsequent release of the viral ribonucloproteins (vRNPs) into the cytosol is unknown for influenza B viruses, it was speculated that influenza B virus vRNPs might be released in the early endosomes, whereas influenza A virus vRNP complexes are released in the late endosomes [48], resulting a more rapid activation of the innate immune system. The late release of vRNP complexes into the cytosol by influenza A viruses and thereby the delay in activation of the innate immune system might be explained as an immune evasion strategy.

Influenza viral proteins can contribute directly to antagonizing the antiviral innate immune response, especially the NS1 protein. Cells infected with genetically

modified influenza viruses with a non-functional NS1 gene displayed stronger IFN responses than cells infected with wild type virus. Viruses with a NS1 defect also display reduced virulence after infection of mice and pigs [353-359]. NS1 inhibits RIG-I receptor signaling by various means. One way the influenza A virus NS1 protein (A/NS1) antagonizes the IFN response is by binding to viral ssRNA and dsRNA in order to prevent RIG-I and PKR activation [173, 360]. Influenza B virus NS1 protein (B/NS1) is able to partially antagonize the IFN response by binding to dsRNA and thereby inhibiting PKR activation [361-363]. Whether B/NS1 can also bind ssRNA is currently unknown. More downstream of the RIG-I signaling pathway, A/NS1 prevents oligomerization of TRIM25 by interacting with the coiled coil domain, and so inhibits TRIM25-mediated RIG-I CARD ubiquitination which is essential for downstream signaling [364], a function currently unknown for B/NS1. Activation and nuclear translocation of IRF-3, NF-κB and ATF-2/c-Jun is also prevented by A/NS1 [365-368]. Hereby A/NS1 limits RIG-I mediated transcriptional activation of the IFN-β promoter [369, 370]. So far, B/NS1 has only been shown to inhibit nuclear translocation of IRF-3 [371]. A/NS1 also alters host cell gene expression by binding to CPSF30 (cleavage and polyadenylation specificity factor); it prevents polyadenylation of the 3' end of host pre-mRNA [372-374]. Furthermore, A/NS1 limits gene expression in general, interfering with the mRNA export machinery [375, 376]. Both functions are unknown for B/NS1. Another function unique for the A/NS1 protein is its ability to activate phosphatidylinositol 3-kinase (PI3K) in order to prevent premature apoptosis during viral propagation [377].

NS1 is not the only viral protein that restrains the innate immune system. As for the other viral proteins (PB2, PB1-F2, PA-X and NP) which have been described to have a role in viral evasion of the innate immune response only the cap-snatching mechanism of the polymerase complex has also been described for influenza B viruses [378, 379]. Cap-snatching of host mRNAs reduces host cell gene expression including that of IFN-β [380-384].

Both influenza A virus PB2 (especially variants containing an aspartic acid at position 9) and PB1-F2 (only variants containing a serine at position 66) limit the production of IFN-β through association with MAVS [32, 33, 385-387].

The recently discovered PA-X viral protein is able to repress cellular gene expression, especially those genes involved in regulating the initiation of the cellular immune response [36].

As described above, influenza virus infection leads to the production of antiviral PKR (Table 2). In order for PKR to limit viral replication, it first needs to be activated by viral dsRNA. PKR activation is under tight regulation of the cellular $p58^{IPK}$ protein which inhibits PKR activity, but is inactive when it forms a complex with heatshock protein 40 (hsp40) [388, 389]. Binding of influenza A virus NP to the $p58^{IPK}$-hsp40 complex releases $p58^{IPK}$, and thereby NP inhibits the effects of PKR [390]. In contrast, the influenza A and B virus M2 protein, which also binds the $p58^{IPK}$-hsp40 complex,

inhibits p58^{IPK} release and thereby limits protein synthesis which eventually leads to host cell apoptosis, possibly enhancing viral particle release (figure 6) [391].
In addition to limiting the production of type I IFNs, influenza A virus also disturbs type I IFN receptor signaling. Influenza A virus infection induces the expression of SOCS (suppressor of cytokine signaling) proteins which inhibit IFN α/β receptor signaling on the level of JAK/STAT activation [392, 393].
Besides interfering with innate signaling, influenza A viruses are also able to counteract cells of the innate immune system. For example, influenza A virus infection of monocytes impairs their ability to differentiate into mature DCs [394]. Furthermore, it was shown that A/NS1 can inhibit DC maturation, and so indirectly limit the induction of virus-specific CD8$^+$ T cell responses [395]. The NK response elicited during an infection is also evaded by the influenza A virus [396]. The gradual mutation of glycosylation sites of influenza virus HA proteins leads to reduced NK recognition of the HA on virus-infected cells [397]. Downregulation of the ζ chain of NKp46 receptors by free HA proteins results in impaired signaling and thereby decreased cytotoxicity of NK cells [398]. Furthermore, influenza A virus can directly infect and kill NK cells [399].

Escaping the humoral immune response

Various mechanisms contribute to immune evasion of influenza viruses from the humoral immune response. Due to the lack of proofreading activity, the transcription of viral RNA by the viral RNA polymerase is error prone and results in mis-incorporation of nucleotides. As a result, quasi species of viruses are formed with random mutations in the genome. The nucleotide substitution rate for both influenza B lineages is estimated around 2.0 x 10^3 substitutions per site per year and is considerably lower than the estimates for seasonal A/H3N2 (5.5 x 10^3) and A/H1N1 (4.0 x 10^3) viruses [67, 70, 400, 401]. Under the selective pressure of antibodies that are present in the human population, induced after influenza virus infections and/or vaccination, variants are positively selected from the quasi species that have accumulated amino acid substitutions in the antigenic sites of HA that are recognized by virus-neutralizing antibodies. This antigenic drift allows the virus to evade recognition by antibodies and to cause recurrent influenza epidemics yearly (Figure 3) [68, 69, 79, 400].
The introduction of an influenza A virus of a novel antigenically distinct subtype into the human population (antigenic shift) may cause a pandemic outbreak when successful human-to-human transmission is established, since neutralizing antibodies against the new virus strain are absent in the population at large (Figure 3) [79]. Introduction of antigenically distinct viruses can occur after zoonotic transmission of influenza A viruses only, as influenza B viruses lack an animal reservoir. However, in most cases, pandemics were caused by viruses that had exchanged gene segments between human and avian or swine influenza A

viruses [86, 402]. For re-assortment to take place, cells need to be infected with two influenza A viruses simultaneously [155]. Since epithelial cells of the swine respiratory tract have receptors for both avian and human influenza A viruses, this species can serve as a mixing vessel for the emergence of re-assorted influenza A viruses [85, 111, 403-405]. Of interest, functionally important and conserved sequences in the surface proteins, like the fusion peptide, are inaccessible for antibody recognition, since they are buried inside the protein [406]. Similar strategies to evade antibody recognition are shared by other viruses, like human immunodeficiency virus (HIV) [407, 408].

Escaping the cellular immune response

Viruses have adopted various strategies to evade recognition by virus-specific T cells. For example, viruses with a large DNA genome (e.g. herpes viruses) can encode proteins that interfere with various steps in the antigen processing and presentation pathways [409]. Most RNA viruses, including influenza viruses, have relatively small genomes and limited coding capacity. Thus far only the PA-X protein has been described to down-regulate MHC class I expression [36]. Influenza A viruses also evade recognition by T cells through their high mutation rates and the selective pressure exerted by virus specific T cells.

Relatively more non-synonymous mutations are observed in the CTL epitope regions of influenza A virus NP than in the rest of the protein, indicating that CTL epitopes are under selective pressure [410]. However, amino acid substitutions flanking an epitope may also affect antigen presentation by changing the cleavage motifs used by the proteasome, alter trimming of the N- and C-terminal sequence by cytosolic or ER resident proteases or impair the translocation via TAP (Figure 8) [411-414] as was demonstrated for several chronic infectious diseases, namely for HIV [415-417], Hepatitis C Virus (HCV) [418] and Epstein-Barr Virus (EBV) [419, 420]. So far, it is unknown whether this CTL evasion mechanism is utilized by viruses that cause acute infections like influenza viruses.

Amino acid substitutions inside CTL epitopes may affect presentation of the epitope in different ways. Amino acid substitutions at an anchor residue may result in complete loss of the epitope, since it may no longer bind to its corresponding MHC class I molecule (Figure 8) [421-425]. Mutations at TCR contact residues can affect recognition by specific T cells, since the epitope no longer matches the specificity of the TCR (Figure 8) [422, 423, 426, 427]. These types of mutations have been observed in escape mutants of viruses that chronically infect their host, like HIV [428-435], HCV [436], EBV [437, 438] and Lymphocytic Choriomenigitis Virus (LCMV) [439]. Both types of amino acid substitutions also have been observed during the evolution of seasonal influenza A/H3N2 viruses [422, 424, 425]. An example of a mutation at an anchor residue is the R384G substitution in the HLA B*2705 restricted influenza A virus $NP_{383–391}$ epitope [424]. This substitution considerably

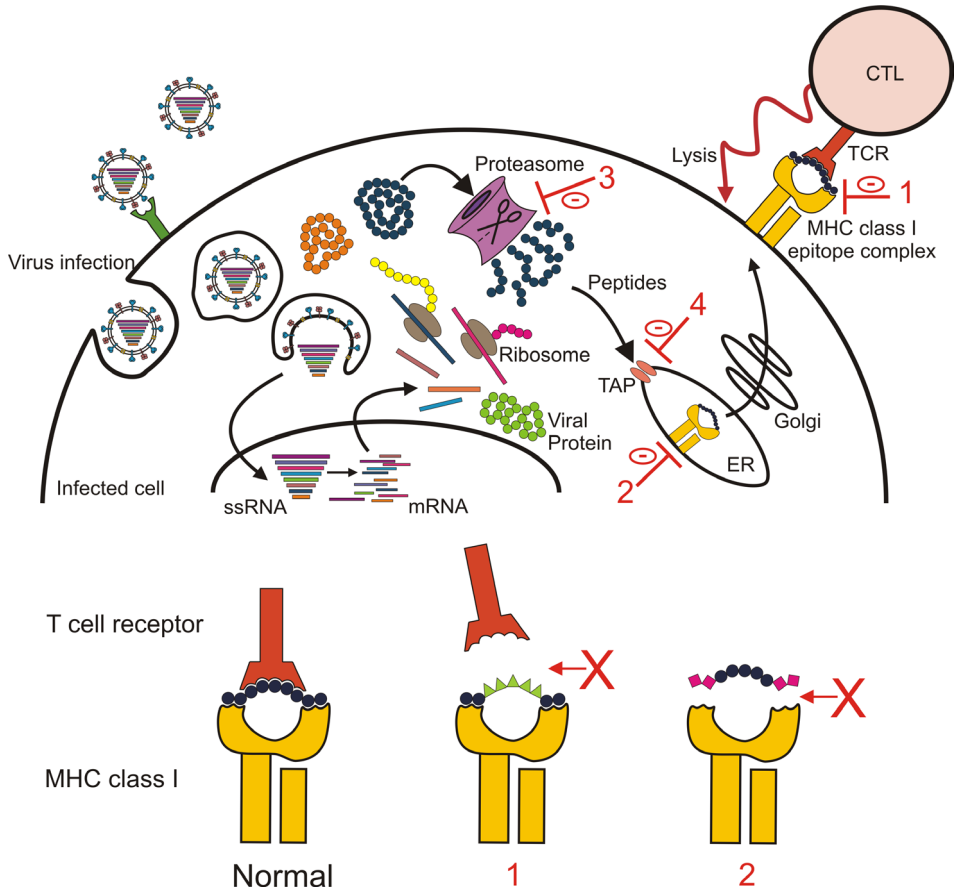


Figure 8 MHC class I presentation of influenza virus epitopes and viral escape
This figure represents a virus-infected cell and the presentation of viral epitopes by MHC class I molecules. The virus can escape recognition by virus specific CTLs by: (**1**) Mutations in TCR contact residues of CTL epitopes in order to prevent recognition of the epitope MHC class I complex by specific CTLs, or (**2**) mutating the anchor residues of the CTL epitope which prevents binding of the epitope to MHC class I molecules. Furthermore, mutations outside the CTL epitope may affect antigen processing by the proteasome or transport via the TAP respectively (**3** and **4**).

affected the human virus-specific CTL response *in vitro* [421]. It is remarkable that this mutation reached fixation rapidly, despite the fact that it is recognized by a minority of human subjects only. This could be explained by strong intra-host advantages and founder effects in a theoretical model [440].
An example of amino acid variation at TCR residues includes that observed in the HLA-B*3501 restricted influenza A virus $NP_{418–426}$ epitope [426, 427]. Variation in this epitope displays signs of antigenic drift [441], and dictates the specificity of the CTL response to this epitope and also forms an explanation for cross reactivity of CTL against contemporary viruses with historic strains [442].
Of interest, functional constraints may limit variation in CTL epitopes. For example, the R384G mutation in the $NP_{383–391}$ epitope was detrimental to viral fitness

and was only tolerated in the presence of functionally compensatory co-mutations [443, 444]. Other influenza A CTL epitopes remain highly conserved even between different subtypes of influenza A viruses, like the HLA-A*0201 / HLA-C*0801 restricted $M1_{58-66}$ epitope [445-447]. The conservation of this epitope remains high despite the immunodominant nature of the epitope [313, 448] and the high prevalence of the HLA-A*0201 allele in the human population [449]. Mutations at the TCR contact or anchor residues are simply not tolerated without loss of viral fitness [410, 450], most likely because of a highly conserved nuclear export signal that overlaps with the $M1_{58-66}$ epitope [451, 452]. The virus' inability to mutate this epitope is remarkable, especially since its origin in currently circulating seasonal A/H3N2 viruses can be traced back all the way to great pandemic of the 1918s (Figure 4) [81-83, 86, 91, 101, 453, 454]. At present, it is unknown if influenza viruses can also accumulate extra-epitopic mutations in order to prevent efficient processing and presentation of these highly conserved epitopes.
Of interest, amino acid variation in the HA of influenza A/H3N2 viruses was also associated with escape from recognition by $CD4^+$ T cells, but not with escape from recognition by antibodies [455].
Thus far, no studies were performed to assess whether influenza B viruses are capable of escaping the influenza B virus-specific $CD4^+$ T cell and CTL responses.

Influenza vaccines

To prevent severe disease and mortality, annual vaccination of individuals at high risk for influenza is recommended [65].

Current influenza vaccines

Trivalent versus quadrivalent vaccines

Trivalent seasonal influenza vaccines have been the gold standard for many years. These vaccines contain components of two influenza A strains (seasonal influenza A/H3N2 and A/H1N1 strain) and one influenza B strain (B/Yamagata or B/Victoria lineage) [456]. Unfortunately, antibodies directed against a virus of one influenza B lineage poorly cross-react with viruses of the opposing influenza B lineage [457, 458]. Since only one influenza B virus lineage is included, vaccine effectiveness is reduced in case the epidemic strain is of the opposing lineage [457, 459, 460]. The increased co-circulation of both influenza B lineages in the last decade has led to more frequent mismatches between the vaccine strain and the most dominant circulating influenza B lineage [70, 159, 457, 461-463]. Notably, in the ten seasons between 2001-2002 and 2010-2011, the B lineage selected for the seasonal trivalent influenza vaccine only matched the dominant circulating B lineage 5 times in the US and Europe [159, 461]. Frequent mismatches were also observed for the southern hemisphere. In Australia 7 out of 13 and in New Zealand 8 out of 13 seasons (2001 to 2013) were (partially) mismatched [70]. To address the

problem of two co-circulating antigenically distinct influenza B lineages, quadrivalent vaccines, containing components of both influenza B lineages, have become available in some countries [456, 464, 465]. Clinical trials thus far have shown that quadrivalent vaccines are comparable to trivalent vaccines regarding safety and immunogenicity [466-475]. Recommendations for virus strains used for next years' quadrivalent vaccines can be found on the WHO website [456]. The benefits of using a quadrivalent vaccine over a trivalent vaccine are obvious, as they elicit antibody responses against viruses of both lineages [466-469], they eliminate the risk that the incorrect influenza B lineage is selected for inclusion in the vaccine. However, unforeseen antigenic drift within either influenza B lineage or the two influenza A virus strains may still affect vaccine effectiveness.

Inactivated versus live attenuated vaccines

Currently used seasonal influenza vaccines are predominantly inactivated vaccine preparations that aim at the induction of strain-specific antibodies that match the epidemic strains [476, 477]. As described above, antigenic drift of influenza viruses allows the seasonal viruses to escape the neutralizing activity of antibodies induced by previous infections or vaccination. Therefore, the vaccine fails to afford life-long protection and needs to be updated almost annually [478]. Furthermore, the production of the vaccine takes several months, so the recommendation for the vaccine strains of the upcoming influenza season is made months in advance [478]. In most influenza seasons, the predicted vaccine strains match the epidemic strains. Occasionally however, a predicted influenza vaccine strain does not match the circulating strain, resulting in suboptimal protection afforded by the vaccine [479-481]. In the event of an emerging pandemic outbreak, the time needed to produce and distribute a pandemic influenza vaccine is also a major drawback [482-484]. Seasonal influenza vaccines do not afford protection against pandemic strains of novel subtypes, since the vaccine-induced antibodies do not cross-react and cross-reactive T cell responses are not induced. Currently-used inactivated vaccines may even interfere with the induction of cell-mediated immunity otherwise induced by natural infections, especially in young children that are still immunologically naïve to influenza viruses. In this way, inactivated vaccines can hamper the development of heterosubtypic immunity [485-490].

Alternatively, cold-adapted live attenuated seasonal influenza vaccines are used [491-494]. The advantage of live attenuated vaccines is that they also elicit cellular immune responses [495, 496] and mucosal immunity [493]. In addition live attenuated seasonal influenza vaccines have been shown to be more effective in preventing influenza virus infections in young children as compared to inactivated vaccines [497, 498]. Also quadrivalent live attenuated influenza vaccines are available [499]. Unfortunately, the administration of live attenuated influenza vaccines is restricted for certain high-risk groups [464, 500].

Novel vaccine strategies

Ideally, seasonal vaccines are used that induce broad-protective immunity against drift variants and potentially pandemic viruses of novel subtypes. The development of vaccines that induce broadly neutralizing antibodies and preferably long-lasting heterosubtypic CTL responses is desirable.

Current influenza vaccines may benefit from adjuvants that aim to induce stronger T cell responses. Although adjuvants that are currently available, like aluminium salts ($Al(OH)_3$ and $AlPO_3$) and oil-in-water adjuvants (e.g. MF59 and ASO3) enhance antibody responses they lack the ability to induce broad-reactive CTL responses [501-505]. Unfortunately, the ASO3-adjuvanted 2009 pandemic A/H1N1 vaccine was associated with an increased risk of narcolepsy in children [506].

Since viral proteins like NP and M1 are highly conserved, they are likely targets for the induction of cross-reactive T cell responses [507]. Induction of efficient CTL responses depends on effective endogenous antigen processing and presentation by MHC class I. This requires effective delivery of viral proteins into the cytosol. Several cytosolic delivery vaccine candidates are under investigation, including DNA vaccines, recombinant viral vectors, ISCOMS and virosomes, some of which already have made it into clinical trials [508-516].

Also, the induction of cross-reactive antibodies has attracted attention in recent years; antibodies directed against the more conserved stem region of the HA molecule are of special interest. In contrast to the subtype-specific antibodies induced against the globular head of HA, these HA stem-specific antibodies display broad-neutralizing activity against multiple influenza virus subtypes [243, 244, 252]. Using this stem region as an immunogen, broadly protective antibody responses could be induced [247, 517-519]. Another broad-protective vaccine approach targets the highly conserved HA_0 cleavage site of influenza B viruses [520].

Other vaccine strategies aim at the induction of antibody responses to the more conserved M2 surface protein. The ectodomain of the M2 protein is highly conserved and antibodies induced against this region afford protection against challenge infection [266, 269, 270, 521, 522]. The mode of action is not neutralization *per se*, since M2 is a minor antigen on virus particles. However, since it is also expressed on the surface of infected cells, ADCC is probably responsible for the protective effect of these antibodies [268].

Recently, live attenuated influenza vaccines have been developed by disrupting NS1 activity through deletion or truncation of the NS1 gene [356, 358, 359, 367, 523-525]. Hence, the virus is unable to interfere with the innate immune response, leading to stronger immune responses after vaccination.

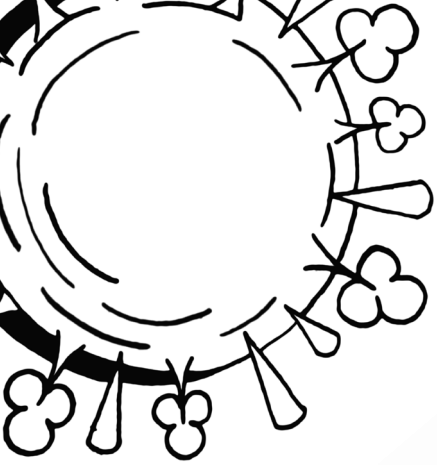
Outline of this thesis

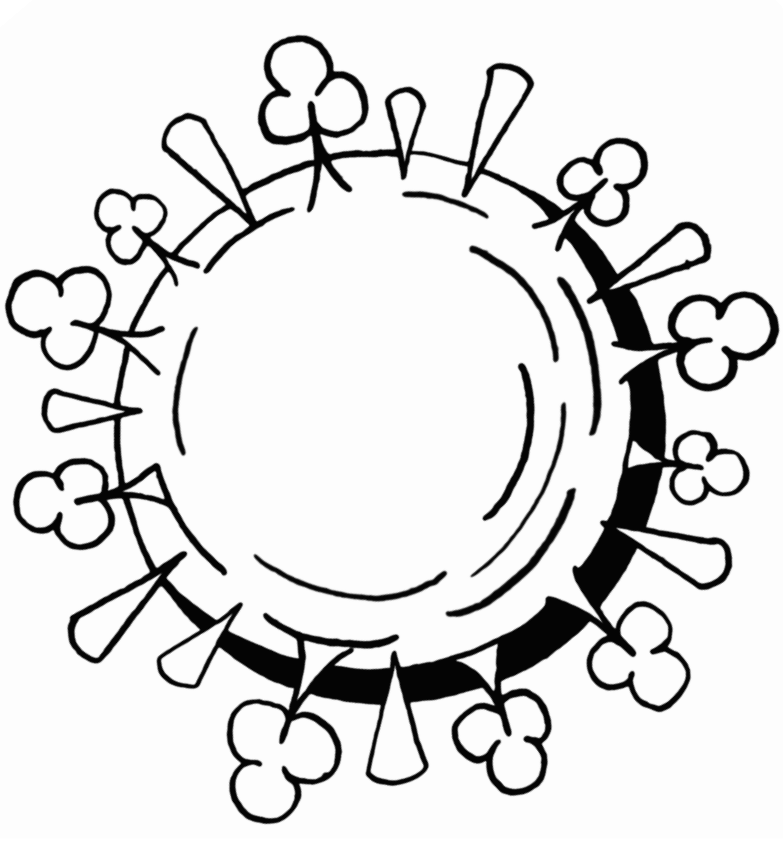
In this thesis, the role of human CD8$^+$ cytotoxic T lymphocytes (CTLs) in the formation of long-lasting broad protective heterosubtypic immunity was assessed.

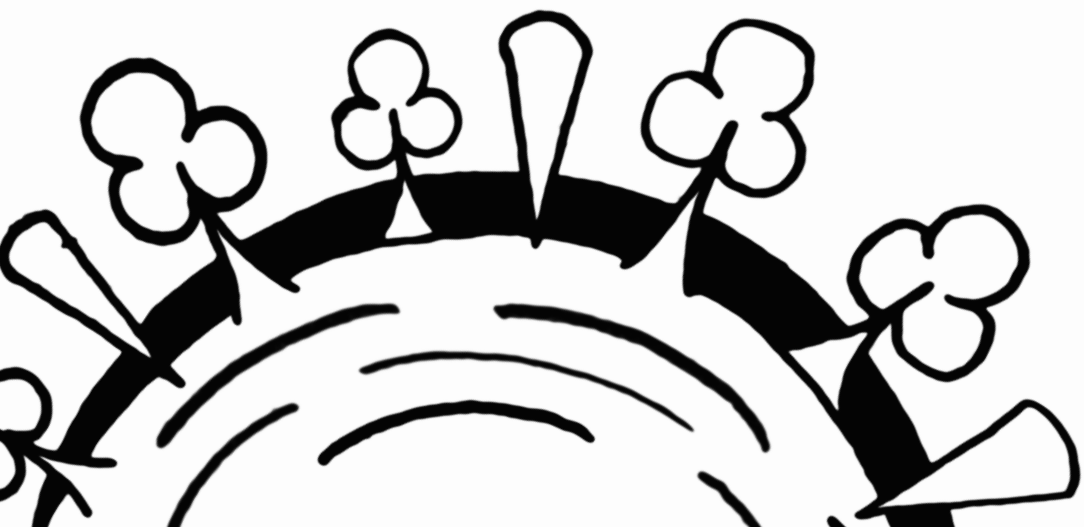
Although the role of CTLs and their target epitopes has been intensively studied for influenza A virus infections, strikingly little is known about the role of CTLs in (cross-) protective immunity against influenza B viruses. This is of special interest because influenza B virus neutralizing antibodies poorly cross-react with viruses of the opposing lineage. Up to now only a handful of influenza B virus CTL epitopes have been described in literature, but possible cross-reactivity of these CTLs has not been studied. To assess the cross-reactivity of influenza B virus-specific polyclonal CTLs, obtained from healthy study subjects, with intra-lineage drift variants and viruses of the opposing lineage we determined their interferon gamma (IFN-γ) response and lytic activity after stimulation with cells infected with the opposing lineage. In chapter 2 we demonstrate for the first time that CTLs directed to viruses of the B/Victroia lineage cross-react with viruses of the B/Yamagata lineage and vice versa. In chapter 3, we assessed the cross-reactivity of human CTLs specific for seasonal influenza A viruses with the newly emerging A/H7N9 virus. Seasonal influenza A H3N2, H1N1 and H1N1pdm09 virus-specific polyclonal CD8$^+$ T cells, obtained from healthy study subjects, displayed a strong IFN-γ response and lytic activity to A/H7N9 virus-infected target cells. The level of recognition was similar to that of seasonal human influenza A virus infected target cells. It was concluded that CTLs specific for seasonal influenza A viruses are cross-reactive with the novel A/H7N9 virus. Thus, pre-existing CTL immunity to seasonal influenza A viruses in the human population may provide a certain level of protection against potentially pandemic heterosubtypic influenza A viruses, like A/H7N9.
However, the longevity of these (cross-reactive) influenza virus-specific CTL responses is largely unknown. In chapter 4 we used peripheral blood mononuclear cells (PBMCs), obtained from healthy blood donors between 1999 and 2012, to assess the longevity of the influenza A virus-specific CTLs. We were able to demonstrate that influenza A virus-specific CTLs are long-lived and that the various subsets were relatively stable over the years. Furthermore, intercurrent influenza A virus infections transiently increased the frequency of functional distinct subsets of influenza A virus-specific CTLs, in particular the effector, effector memory and effector memory RA subsets. These subsets decreased in the contraction phase to "baseline" levels. Since the majority of these CTLs are cross-reactive they are likely to provide protective immunity against an antigenically distinct influenza A virus (like A/H7N9) even in the absence of a recent seasonal influenza A virus infection.
Although the internal proteins against which most CTLs are directed are generally more conserved than the antigenic sites of neutralizing antibodies, CTL epitopes have previously been shown to be variable under specific CTL immune

pressure. However, some influenza A virus CTL epitopes are highly conserved between different subtypes of influenza A viruses. They do not allow mutations in their T cell receptor contact or MHC class I anchor residues without loss of viral fitness. In chapter 5, we investigate the role of extra-epitopic amino acid mutations in the CTL escape of the otherwise fully conserved HLA-A*0201 restricted $M1_{58-66}$ (GILGFVFTL) epitope. We were able to demonstrate that naturally acquired amino acid substitutions in extra-epitopic positions of the human seasonal A/H3N2 virus resulted in a delay and reduced activation of $M1_{58-66}$-specific CTLs when compared to stimulation with cells infected with an avian A/H5N1 virus in which these amino acid substitutions were absent. We are the first to demonstrated that naturally occurring extra-epitopic substitutions in human influenza A viruses play a role in evading the virus-specific CTL response. The absence of these substitutions in the 2009 influenza A virus pandemic might have contributed to an overall less severe disease outcome for individuals carrying the HLA-A*0201 allele.
Based on these and other studies we conclude that CTLs contribute to protective immunity against severe influenza A virus infection especially in the absence of a protective antibody response for example during a pandemic outbreak. Seasonal inactivated influenza vaccines fail to induce an antibody response that cross-reacts with potentially pandemic viruses like A/H5N1 and A/H7N9. In addition these vaccines induce a broadly-reactive CTL response inefficiently. They may even hamper the induction of cross-reactive CTLs that would have otherwise been induced by natural influenza A virus infections. In chapter 6, we have tested an novel adjuvant, G3/DT, for its ability to induce CTL responses after vaccination with a traditional trivalent inactivated influenza vaccine in a mouse model. The adjuvant strongly enhanced the virus-specific antibody response to all three vaccine strains and in addition promoted the induction of a virus-specific $CD8^+$ T cell response. The induction of these CTLs correlated with protection against a lethal infection with an antigenically distinct influenza A virus.

Collectively, the results presented in this thesis illustrate that potentially cross-reactive CTLs are long-lived in human subjects. Moreover, CTLs directed against the highly conserved immunodominant $M1_{58-66}$ epitope might even respond more strongly to avian influenza A viruses deprived of extra-epitopic CTL escape substitutions. Adjuvants that aim to induce these cross-reactive CTL responses after vaccination with traditional influenza vaccines may reduce disease severity in case of an emerging pandemic with an antigenically distinct influenza virus. Potential implications of these studies are discussed in chapter 7.







CHAPTER 2:

Influenza B virus-specific CD8$^+$ T-lymphocytes strongly cross-react with viruses of the opposing influenza B lineage

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Abstract

Influenza B viruses fall in two antigenically distinct lineages (B/Victoria/2/1987 and B/Yamagata/16/1988 lineage) that co-circulate with influenza A viruses of the H3N2 and H1N1 subtype during seasonal epidemics. Infections with influenza B viruses contribute considerably to morbidity and mortality in the human population. Influenza B virus neutralizing antibodies, elicited by natural infections or vaccination, poorly cross-react with viruses of the opposing influenza B lineage. Therefore, there is an increased interest in identifying other correlates of protection which could aid the development of broadly-protective vaccines. BLAST analysis revealed high sequence identity of all viral proteins. With two online epitope prediction algorithms, putative conserved epitopes relevant for study subjects used in the present study, were predicted. The cross-reactivity of influenza B virus-specific polyclonal $CD8^+$ T lymphocyte populations, obtained from HLA-typed healthy study subjects, with intra-lineage drift variants and viruses of the opposing lineage was determined by assessing their *in vitro* interferon gamma (IFN-γ) response and lytic activity. Here, we show for the first time, to the best of our knowledge, that CTLs directed to viruses of the B/Victoria lineage cross-react with viruses of the B/Yamagata lineage and vice versa.

Introduction

Influenza A viruses of the H1N1 and H3N2 subtypes and influenza B viruses cause annual outbreaks of respiratory tract disease in humans [65]. Seasonal recurrence of these viruses is a result of selection of variants that evade recognition by virus neutralizing antibodies induced by previous infections or vaccination (antigenic drift) [67-69]. In contrast to influenza A viruses, influenza B viruses are not further subdivided in antigenically distinct subtypes. Also the host range of influenza B viruses is more limited [526] than that of influenza A viruses, which infect a wide range of animal species and for which aquatic birds constitute a major reservoir of most subtypes [527]. Since animal influenza A viruses of various subtypes have been shown to be able to cross the species barrier and can cause pandemic outbreaks, they continue to pose a threat for public health [138, 528].

Although no subtypes of influenza B viruses have been identified, two antigenically distinct lineages are distinguished based on their hemagglutinin (HA); the B/Victoria/2/1987 (B/Vic) and B/Yamagata/16/1988 (B/Yam) lineages [6]. The B/Yam-lineage was the dominant lineage until the mid-1980s when B/Vic made a global appearance after its earlier detection in the 1970s in China [158]. Viruses of both influenza B lineages co-circulated with influenza A/H3N2 and A/H1N1 viruses during various seasonal epidemics [159, 529, 530]. However, the relative contribution of these types and subtypes of influenza virus to the respective epidemics varies over the years. Influenza B viruses can be the major cause of seasonal epidemics or almost be completely absent [6, 73, 159, 463].

Overall, influenza A/H3N2 virus infections are associated with highest disease severity, however infections with influenza B virus contribute considerably to morbidity and mortality in the human population [73-75, 159, 531]. Although influenza B viruses cause disease in all age groups, the burden of influenza B virus infections is highest among children and young adults [159, 463, 532]. To prevent severe disease and mortality, annual vaccination of individuals at high risk for influenza is recommended [65]. For many years trivalent seasonal influenza vaccines have been used. These vaccines contain components of three strains that match circulating influenza viruses antigenically, influenza A/H3N2 and A/H1N1 and one influenza B strain of either the B/Yam or B/Vic -lineage [533]. These vaccines aim at eliciting virus neutralizing strain-specific antibody responses. Unfortunately, antibodies directed against a virus of one lineage of influenza B poorly cross-react with viruses of the opposing influenza B lineage [457, 458]. The time-consuming process of vaccine production requires recommendation of vaccine strains months in advance of the upcoming influenza season [478]. Since only one strain of influenza B virus is included in most current trivalent seasonal influenza vaccines, vaccine effectiveness is reduced in case the epidemic strain is of the opposing lineage [457, 459, 460]. The increased co-circulation of both influenza B lineages in the last decade has led to more frequent mismatches

between the vaccine strain and the most dominant circulating influenza B lineage [159, 457, 461-463]. To address this problem, quadrivalent influenza vaccines, containing components of both influenza B lineages, have become available in some countries [464, 465, 533]. However, vaccine effectiveness may still be reduced in case of unforeseen antigenic drift within either influenza B lineage [457]. This spurred an increased interest in identifying other correlates of protection, which could be relevant for future developments of broadly-protective vaccines [528]. Of interest, antibodies cross-reactive with viruses of both the B/Yam and B/Vic lineage have been demonstrated, but they contribute to a limited extent to the overall antibody repertoire [252]**.** In the present paper we investigate the cross-reactivity of influenza B virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs). The main function of CTLs is to detect and eliminate virus infected cells, thereby restricting viral replication and accelerate viral clearance [344, 528]. Numerous studies have demonstrated that influenza A virus-specific CTLs contribute to heterosubtypic immunity against antigenically distinct influenza A virus strains. Influenza A virus-specific CTLs are predominantly directed to more conserved internal proteins [311, 315-317, 534-537] and their contribution to cross-protective immunity has been demonstrated in various animal models [337-339, 538-540]. Although *in vivo* evidence for the role of CTLs in protective heterosubtypic immunity in humans is limited [344-347], several *in vitro* studies have demonstrated that human CTLs directed to seasonal influenza A viruses cross-react with possible pandemic influenza A viruses, including avian influenza viruses of the H5N1 and H7N9 subtype and swine origin vH3N2 viruses [316, 317, 535-537, 541]. Also after influenza B virus infections, virus-specific CTLs are induced [542-544], but it is unknown to what extent human CTLs directed to an influenza B virus of one lineage can cross-react with viruses of the opposing lineage. Here, we show for the first time, to the best of our knowledge, that polyclonal $CD8^+$ T cells directed to influenza B viruses of the B/Vic-lineage can cross-react with viruses of the B/Yam-lineage and vice versa, although the antigen-specificity of these cross-reactive $CD8^+$ T cells was not defined. Furthermore by using the prototypic viruses of both lineages (B/Victoria/2/1987 and B/Yamagata/16/1988) and more recent descendants (B/Netherlands/455/2011 and B/Netherlands/712/2011 respectively) we showed that these $CD8^+$ T cells also recognize intra-lineage drift variants.

Materials and Methods

Cells

PBMCs were obtained from nine HLA-typed healthy blood donors (18-64 years of age) (Sanquin Bloodbank, Rotterdam, the Netherlands) by means of lymphoprep (Axis-Shield PoC, Oslo, Norway) gradient centrifugation and were subsequently cryopreserved at -135°C. Study subjects were divided into three groups based on

their HLA class I alleles (Table 2). The use of PBMCs for scientific research was approved by the Sanquin Bloodbank after informed consent was obtained from the blood donors.

Viruses

Prototypic influenza viruses B/Victoria/2/1987 (Kindly provided by Vicki Gregory, WHO Collaborating Centre for Influenza, National Institute for Medical Research, London) and B/Yamagata/16/1988 as well as the two more recent viruses B/Netherlands/455/2011 and B/Netherlands/712/2011 belonging to either lineage respectively, were propagated in Madin Darby Canine Kidney (MDCK) cells at 37°C. Culture supernatants were clarified by low-speed centrifugation and subsequently concentrated by ultracentrifugation after which their infectious-virus titers were determined as described previously [545] using 96 wells plates (Greiner Bio-One).

Sequence analysis

Sequences of the eight gene segments of the above described viruses were obtained as described previously [546] using segment specific primers. Nucleotide sequences of the HA gene segments were used for phylogeny and amino acid sequences of all gene segments were used to determine the amino acid sequence identity and for epitope prediction analysis as described below.

Phylogeny

Besides the above mentioned viruses an additional 47 human influenza B viruses, either used as vaccine strain or of which the lineage was previously confirmed by means of hemagglutination inhibition assay [457, 461, 533, 547, 548] and of which the complete HA nucleotide sequences was available from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genomes/FLU) or the Global Initiative on Sharing Avian Influenza Data (GISAID; http://gisaid.org) influenza database, were selected for phylogenetic analysis. Previously published HA accession numbers are provided in the supplementary data (Table S1). Nucleotide sequences of the HA gene segment of all 51 human influenza B viruses were aligned using the Clustal W program running within the BioEdit software package, version 7.2.5 [549] and manually edited to maintain the correct reading frame. Nucleotides before the start codon and after the stop codon were removed. The nucleotide sequence alignment was used to determine the best-fit models of nucleotide substitution by jModelTest version 2.1.4 [550, 551]. The preferred ML-optimized model of nucleotide substitution, based on the Akaike information criterion, was TPM1uf+I+Γ4: Kimura 3-parameter model (K81) [552] with unequal base frequencies (uf), the proportion of invariant sites (I) and the gamma distribution of among-site rate variation with four categories estimated from the empirical data (Γ4). ML phylogenetic trees were inferred using the selected model

of nucleotide substitution and the PhyML package, version 3.1 [553], performing a full heuristic search and subtree pruning and regrafting searches. The reliability of all phylogenetic groupings of each tree was determined through a nonparametric bootstrap resampling analysis with PhyML: 1,000 replicates of ML trees were analyzed by applying the TPM1uf+I+Γ4 model of nucleotide substitution. A detailed HA tree, including bootstrap values, is shown in figure 1. Trees were visualized through the FigTree program, version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Amino acid sequence identity

BLAST analysis (http://blast.ncbi.nlm.nih.gov) was used to determine the amino acid sequence identity of all viral proteins of influenza B viruses used in this study, namely B/Victoria/2/1987, B/Yamagata/16/1988, B/Netherlands/455/2011 and B/Netherlands/712/2011 (Table 1).

Immunoinformatic analysis

Epitopes restricted for the HLA-alleles of the study subjects were predicted based on the amino acid sequence of all internal proteins for B/Victoria/2/1987 and B/Yamagata/16/1988. For robustness we used two online programs, namely Syfpeithi (http://www.syfpeithi.de) and Immuneepitope (http://tools.immuneepitope.org/mhci). In the Syfpeithi program, the MHC (HLA) type of the study subjects was selected in combination with prediction of epitopes of all possible lengths (all mers). The program indicated that putative epitopes are among the top 2% of all top-scoring possibilities. An additional cut-off was set at a score of ≥19 since all known influenza A epitopes for these HLA-alleles scored 19 or higher. For the Immuneepitope program we also selected the HLA-alleles of the study subjects in combination with all possible epitope lengths. The program indicated that most putative epitopes have an ANN IC_{50} (nM) score of ≤500 so this was used as a cut-off value. Predicted amino acid sequences of putative epitopes are available on request.

Peptides

The HLA-B*0801 restricted putative epitopes predicted by Immuneepitope and Syfpeithi and present in viruses of both lineages, were ordered as synthetic immunograde peptides (>85% purity) (Eurogentec, Seraing, Belgium), in addition to previously *in vitro* confirmed HLA-B*0801 restricted influenza B epitopes $NP_{263-271}$ ADRGLLRDI and $NP_{413-421}$ ALKCKGFHV [544].

In vitro expansion of influenza B virus-specific CD8⁺ T cells

PBMCs obtained from HLA-typed healthy blood donors were stimulated with B/Victoria/2/1987 or B/Yamagata/16/1988 at a multiplicity of infection (MOI) of 3, as described previously [313]. Polyclonal CD8⁺ T cells were isolated from the expanded PBMC cultures 8 to 9 days after stimulation by means of CD8⁺ magnetically activated cell sorting (MACS) bead sorting according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). These polyclonal CD8⁺ T cells were used as effector cells in IFN-γ ELISpot and lytic assays (see below).

Target cells

HLA-matched BLCLs were prepared as described previously [513]. The cells (10^6) were incubated with or without 10μM peptide for one hour (hr) at 37°C and subsequently washed and resuspended in RPMI 1640 medium (Lonza, Basel, Switzerland) containing antibiotics and 10% fetal bovine serum (Sigma-Aldrich, Zwijndrecht, The Netherlands) (R10F medium). Virus-infected target cells were prepared by inoculating BLCLs at an MOI of 3 with B/Victoria/2/1987, B/Yamagata/16/1988, B/Netherlands/455/2011 or B/Netherlands/712/2011 or left untreated (negative control). After one hr, cells were washed and resuspended in R10F medium and cultured for 16 to 18 hr at 37°C before being used for the stimulation of T cells in IFN-γ ELISpot assays or as target cells in the CTL assays (see below).

IFN-γ ELISpot assay

The IFN-γ responses of *in vitro*-expanded polyclonal CD8⁺ T cells were determined by ELISpot assays, which were performed according to the manufacturer's recommendations (Mabtech, Nacka Strand, Sweden). In brief, 10,000 *in vitro*-expanded polyclonal CD8⁺ T cells were used as effector cells and incubated overnight (o/n) with 30,000 peptide-loaded, virus-infected or untreated HLA class I-matched target cells, in triplicate. The average number of spots was determined using an ELISpot reader and image analysis software (Aelvis, Sanquin Reagents, Amsterdam, The Netherlands).

CTL assay

To examine the lytic capacity of the *in vitro*-expanded polyclonal CD8⁺ T cells a CTL assay was used with CFSE-labeled target cells. In brief, $5x10^6$ HLA class I-matched BLCLs were incubated with 0,6μM CFSE (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 5 min at 37°C. These cells were subsequently inoculated with B/Victoria/2/1987, B/Yamagata/16/1988, B/Netherlands/455/2011 or B/Netherlands/712/2011 at an MOI of 3 for 16 to 18 hr. The infected and CFSE-labeled BLCLs were used as target cells and cocultured with the *in vitro*-expanded polyclonal CD8⁺ effector T cells in E:T ratios of 5, 2.5 and 1.25. After a 3 hr incubation period dead cells were stained

with ToPro3 (Invitrogen, Breda, The Netherlands) for 10 minutes at 37°C. Lysis in the target cell population was determined by flow cytometry using BD FACSDiva software (Becton Dickinson B.V., Breda, The Netherlands). Experiments were performed in triplicate. Percentage lysis was calculated by the following formula: 100-(100*(viable target cells in sample in presence of effector cells / viable target cells in absence of effector cells)).

Results

Virus characterization

Phylogenetic analysis was performed to confirm the lineage of the influenza B viruses used in the present study. A data set comprising the HA nucleotide sequences of 51 influenza B viruses isolated between 1987 and 2013 was used to determine the lineage of influenza viruses B/Netherlands/455/2011 and B/Netherlands/712/2011. An Maximum likelihood (ML) phylogenetic tree was inferred to study the nucleotide evolution of the HA gene segment of the influenza B Yamagata and Victoria lineage.

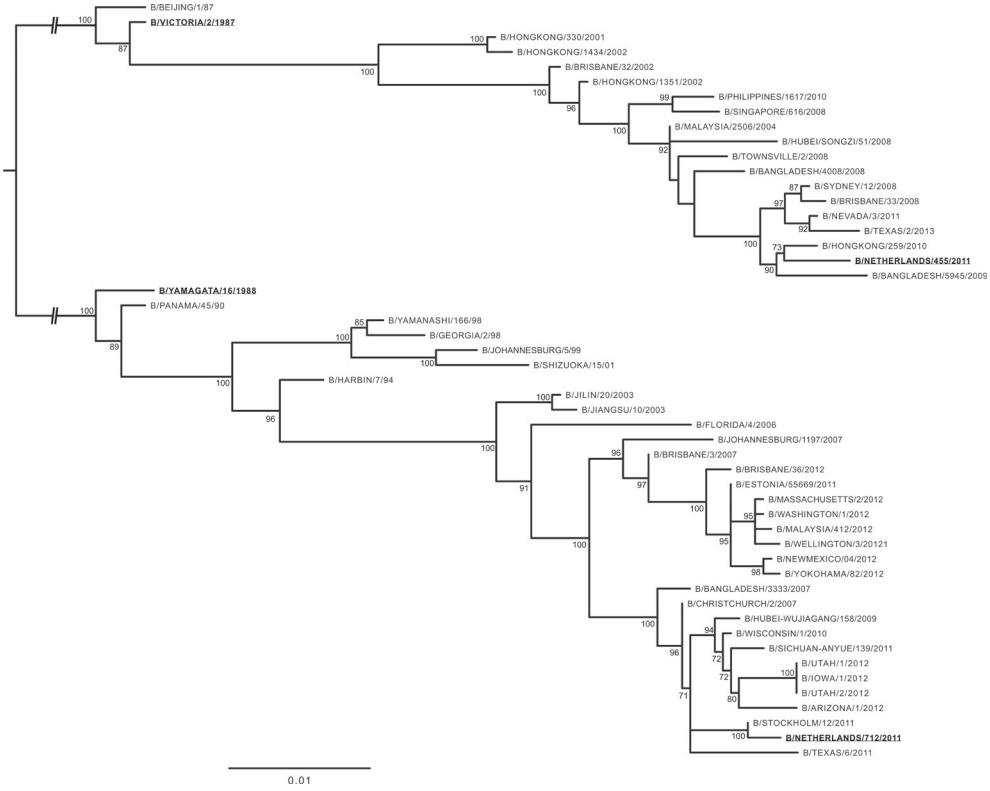

Figure 1 Phylogenetic maximum likelihood tree of the HA gene segment of human influenza B viruses.
The phylogenetic ML tree was inferred from 51 HA nucleotide sequences of both influenza B virus lineages. Bootstrap values of 1,000 replicates of ML trees are shown as percentages (values <70% are omitted). Scale bars roughly represent 1% of nucleotide substitutions between close relatives. Bold underlined sequences were used in the present study.

As expected, both prototypic strains B/Victoria/2/1987 and B/Yamagata/16/1988 are located near the base of the respective lineage. Based on this ML phylogenetic tree, it was concluded that the B/Netherlands/455/2011 virus belongs to the B/Vic lineage while B/Netherlands/712/2011 belongs to the B/Yam lineage (Fig 1).

High amino acid sequence identity between both influenza B lineages

The percentage amino acid sequence identity of influenza A viral proteins has proven to be a good predictor for cross-reactivity of virus specific T cells with influenza A virus of various subtypes [536]. Therefore, we wished to compare the overall amino acid sequence identity between the influenza B viruses used in the present study. BLAST analysis revealed that the sequence identity of all viral proteins was remarkably high (≥86% up to 100%) (Table 1).

Table 1 Percentage amino acid sequence identity between internal proteins of the influenza B viruses used in this study

Virus	Gene segment	Identity (%)		
		B/Victoria/ 02/1987	B/Netherlands/ 712/2011	B/Netherlands/ 455/2011
B/Yamagata/16/1988	PB2	99	99	99
	PB1	99	99	99
	PA	98	99	98
	HA	95	96	95
	NP	98	99	99
	NA	97	95	94
	NB	90	91	90
	M1	100	99	100
	BM2	93	98	96
	NS1	99	94	93
	NS2	100	98	98
B/Victoria/02/1987	PB2		99	99
	PB1		99	99
	PA		98	98
	HA		94	97
	NP		98	98
	NA		93	93
	NB		87	86
	M1		99	100
	BM2		91	89
	NS1		94	93
	NS2		98	98
B/Netherlands/712/2011	PB2			99
	PB1			99
	PA			99
	HA			93
	NP			99
	NA			94
	NB			93
	M1			99
	BM2			96
	NS1			97
	NS2			98

Prediction of conserved CD8⁺ T cell epitopes

CD8⁺ T cell epitope prediction tools Syfpeithi and Immuneepitope were used to predict the presence of putative conserved CD8⁺ T cell epitopes in influenza B viral proteins. Since the amino acid sequence identity between the respective strains was very high, this analysis was performed with the prototypic strains B/Victoria/2/1987 and B/Yamagata/16/1988 only. Epitope predictions were performed for the HLA-alleles that corresponded with those of the study subjects (Table 2). Syfpeithi and Immuneepitope predict a large number of putative epitopes in viral internal proteins of both B/Victoria/2/1987 and B/Yamagata/16/1988 (Fig S1). Due to the large difference in the number of epitopes predicted by both programs we considered an epitope a putative epitope when predicted by both programs. In addition to be a putative conserved epitope, the epitope needed to be present in both viruses (Fig 2). As shown in figure 2, most of the epitopes predicted by both programs were present in both virus strains. Only a small proportion of the predicted epitopes was unique for one of the two viruses. This data suggest that the majority of CTL epitopes are conserved between the two lineages of influenza B.

Table 2 HLA-A and HLA-B haplotypes of the study subjects

Group	Donor	HLA-A and –B haplotypes
I	8904 6888 1578	HLA-A*0101, A*0201, B*0801, B*3501
II	7482 2501 8801	HLA-A*0101, A*0201, B*0801, B*2705
III	6877 9465 5891	HLA-A*0101, A*0301, B*0801, B*3501

To test the robustness of these prediction algorithms we wished to establish *in vitro* the reactivity influenza B virus-specific CD8⁺ T cells with the predicted epitopes. HLA-B*0801 restricted putative epitopes were selected since the HLA-B*0801 was previously shown to be dominant in stimulating influenza B virus-specific CTLs [554] and the HLA-B*0801 allele is present in all the study subjects (Table 2). The HLA-B*0801 allele also gives us the opportunity to use three previously described peptides, namely $NP_{263-271}$(ADRGLLRDI), $NP_{413-421}$(ALKCKGFHV) and NP_{30-38}(RPIIRPATL) [544], of which only the NP_{30-38} epitope was predicted by our prediction algorithms. One study subject of each HLA-group (Table 2; 6888, 8801 and 6877) was selected from which *in vitro*-expanded polyclonal CD8⁺ T cells specific for B/Yamagata/16/1988 were tested for their reactivity with the predicted HLA-B*0801 restricted epitopes using peptide-loaded HLA class I-matched B lymphoblastoid cell lines (BLCLs). To this end, we determined the IFN-γ production of the polyclonal CD8⁺ T cells in an enzyme-linked immunosorbent spot (ELISpot) assay. Although

donor 6877 responded to the NP_{30-38} and $M1_{45-52}$ we did not observe a significant response to any of the other predicted or previously identified epitopes by donor 6877, 6888 and 8801 while all donors had a high response to BLCLs infected with the homologous virus (data not shown). These results clearly indicate that the prediction algorithms are not very reliable and therefore putative epitopes with other HLA restrictions were not tested.

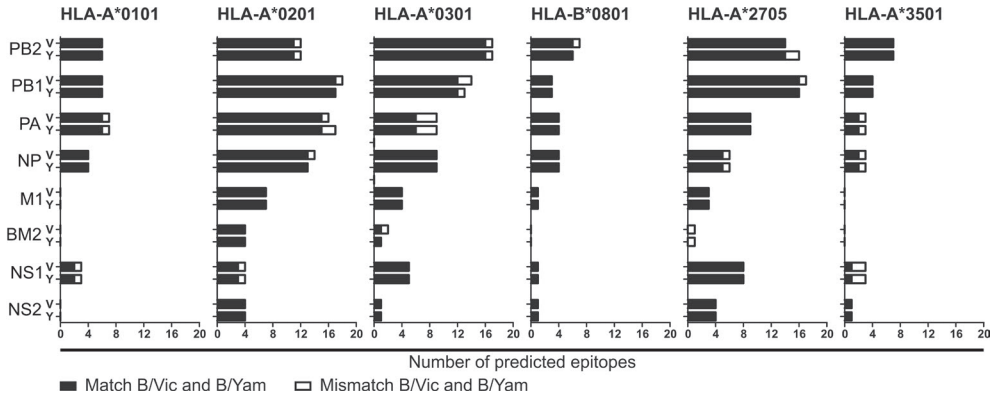

Figure 2 Epitopes predicted by Syfpeithi and Immuneepitope.
Syfpeithi and Immuneepitope algorithms were used to predict putative epitopes. Only epitopes predicted by both algorithms were considered to increase the fidelity of the prediction. Bars represent the total number of putative epitopes for B/Victoria/2/1987 (V) or B/Yamagata/16/1988 (Y). Black bars represent the number of putative conserved epitopes present in both viruses while the white bars represents the number of putative epitopes present in one virus only.

Cross-reactivity of influenza B virus-specific $CD8^+$ T cells assessed by ELISpot

Next, we determined the extent of cross-reactivity of influenza B virus-specific $CD8^+$ T cells with intra-lineage drifted variants and viruses of the opposing lineage. To this end, polyclonal $CD8^+$ T cells derived from B/Victoria/2/1987 or B/Yamagata/16/1988 virus stimulated peripheral blood mononuclear cell (PBMC) cultures were re-stimulated with HLA class I-matched BLCLs infected with the prototypic viruses (B/Victoria/2/1987 and B/Yamagata/16/1988) and the more recent viruses (B/Netherlands/455/2011 and B/Netherlands/712/2011 respectively). Activation of the polyclonal $CD8^+$ T cells was assessed by measuring the number of IFN-γ producing cells per 10,000 $CD8^+$ T cells with the ELISpot assay (Fig 3).
The reactivity of B/Victoria/2/1987 virus-specific $CD8^+$ T cells is shown in figure 3A. These cells of all study subjects responded to reactivation with the homologous B/Victoria/2/1987 virus, although two study subjects (7482 and 2501) were low responders. A similar response was observed after re-stimulation with the intra-lineage drift variant B/Netherlands/455/2011. In addition, after stimulation with a virus of the opposing lineage B/Yamagata/16/1988 or B/Netherlands/712/2011 a cross-reactive response was observed that did not substantially differ in magnitude from the response to viruses of the B/Vic lineage, in most subjects. In figure 3B, the

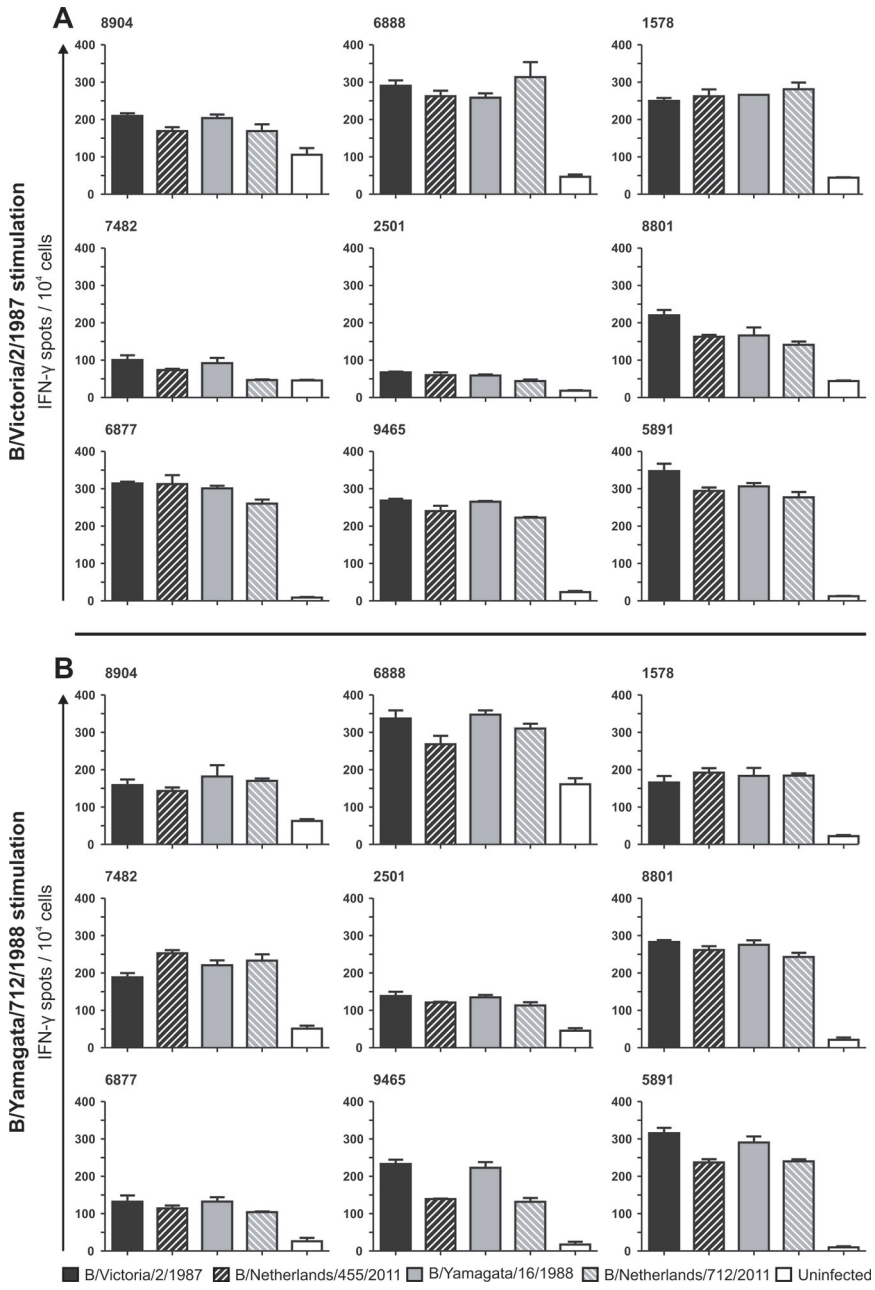

Figure 3 Cross-reactivity of virus-specific polyclonal CD8$^+$ T cells assessed by IFN-γ ELISpot.
B/Victoria/2/1987 virus-specific polyclonal CD8$^+$ T cells (A) or B/Yamagata/16/1988 virus-specific polyclonal CD8$^+$ T cells (B) of 9 study subjects were cocultured with HLA-matched BLCLs infected with B/Victoria/2/1987 (black bars). B/Netherlands/455/2011 (black hatched bars), B/Yamagata/16/1988 (grey bars) or B/Netherlands712/2011 (grey hatched bars). The number of IFN-γ-producing cells per 10,000 polyclonal CD8$^+$ T cells was determined by ELISpot assay. Experiments were performed in triplicate. The error bars indicated standard deviations of the triplicates. Uninfected BLCLs were used as negative controls (white bars). Identification number of the respective study subject is indicated in the left upper corner of each graph.

response of B/Yamagata/16/1988 virus-specific CD8+ T cells is shown. Again, all study subjects responded to the re-stimulation with HLA-matched BLCLs infected with the homologous virus strain and also after stimulation with the intra-lineage drift variant B/Netherlands/712/1988. In addition, a cross-reactive response was observed after stimulation with HLA-matched BLCLs infected with both viruses of the opposing B/Vic lineage (B/Victoria/2/1987 and B/Netherlands/712/2011), which was similar in magnitude compared to the response to viruses of B/Yam lineage.

Cross-reactivity of influenza B virus-specific CD8+ T cells assessed by lytic activity

Next, we wished to assess the cross-reactive lytic capacity of these polyclonal CD8+ T cell populations. Based on the IFN-γ ELISpot results, we selected a strong-responder from each HLA-group of study subjects (6888, 8801 and 9465) to test the lytic capacity of the CD8+ T cells. To this end, *in vitro* expanded B/Victoria/2/1987 or B/Yamagata/16/1988 virus-specific polyclonal CD8+ T cells were incubated with carboxyfluorescein succinimidyl ester (CFSE)-labeled HLA-matched BLCLs infected with B/Victoria/2/1987, B/Yamagata/16/1988, B/Netherlands/455/2011 or B/Netherlands/712/2011. The gating strategy used for flowcytometry to detect lytic activity of the CD8+ T cells is shown in figure 4 A and B.

B/Victoria/2/1987 virus-specific CD8+ T cells displayed lytic capacity against cells infected with the homologous virus and the B/Netherlands/455/2011 virus of the same lineage in a effector-to-target cell (E:T) ratio dependent fashion. In addition, cells infected with the heterologous viruses B/Yamagata/16/1988 and B/Netherlands/712/2011 of the opposing lineage were lysed as well (Fig 4C). A similar pattern of reciprocal lytic activity was observed for the B/Yamagata/16/1988 virus-specific CD8+ T cells (Fig 4D). These results confirmed the cross-reactive nature of influenza B virus-specific CD8+ T cells observed in the ELISpot assay.

Discussion

Influenza B viruses display less antigenic drift than influenza A viruses [67, 157, 555], yet they evade recognition by virus neutralizing antibodies present in the human population efficiently. This necessitates regular updates of the influenza B component of seasonal influenza vaccines. The circulation of influenza B viruses belonging to two antigenically distinct lineages further complicates the production of efficacious influenza vaccines. Inactivated influenza vaccines typically aim at the induction of virus neutralizing antibodies directed to the variable globular head region of the HA molecules of the respective influenza viruses. Consequently, there is interest in protective immune responses directed to more conserved proteins or regions thereof. Understanding humoral and cell-mediated immune responses to these conserved proteins may aid the development of more universal vaccines. Here, we assessed the cross-reactivity of influenza B virus-specific CD8+ T cells with viruses of the opposing lineage. It was concluded that influenza B virus-specific CD8+

T cells display a high degree of cross-reactivity with intra-lineage drift variants and viruses of the opposing lineage.

Although it was beyond the scope of this study to identify novel influenza B epitopes, we were interested whether predicted putative CTL epitopes were conserved between both influenza B lineages. The amino acid sequence identity of all viral proteins was very high between both lineages, which already suggested the existence of cross-reactive T cell epitopes, as was also demonstrated for influenza A viruses [536]. For the *in silico* prediction of epitopes we excluded proteins encoded by the HA and NA gene segments, since these proteins undergo antigenic drift after positive selection by antibodies. Furthermore, it has been shown for influenza A viruses that these envelope proteins are minor targets for CTL responses [317]. Two epitope prediction algorithms that are publically available, Syfpeithi and Immuneepitope, were used in order to predict putative epitopes with the highest possible fidelity. A large number of putative epitopes was predicted by both programs, of which most were present in viruses of both lineages. However, the total number of epitopes predicted by both programs varied widely. The performance of these algorithms is not completely clear and most likely also false positives have been predicted and false negative were omitted [556, 557]. Indeed, out of six *in vitro* confirmed influenza B epitopes described previously [542-544] that corresponded to the HLA-alleles investigated in our study, only the NP_{30-38} RPIIRPATL epitope (HLA-B*0801 restricted) was predicted by both prediction programs. Other epitopes were solely predicted by Immuneepitope (NP_{85-94} KLGEFYNQMM and NP_{85-93} KLGEFYNQM (HLA-A*0201 restricted)) or Syfpeithi ($NP_{413-421}$ ALKCKGFHV (HLA-B*0801 restricted)) and two epitopes ($NP_{263-271}$ ADRGLLRDI (HLA-B*0801 restricted) and NP_{82-94} MVVKLGEFYNQMM (HLA-A*0201 restricted)) were not predicted at all. Since these programs predict epitopes based on binding affinity of the epitope with the selected HLA-allele they do not take into account other possible factors that might play a role, such as the dissociation rate of the epitope [558], folding of the MHC class I molecules [559] or antigen processing [528]. To further test the robustness of the prediction algorithms we determined the reactivity of polyclonal influenza B virus-specific polyclonal $CD8^+$ T cells of three study subjects with the HLA-B*0801 restricted putative epitopes. We were unable to confirm any of the predicted epitopes, which included three previously described HLA-B*0801 restricted epitopes (NP_{30-38}, $NP_{263-271}$ and $NP_{413-422}$). The lack of response to these previous described epitopes may be explained by mis-match of the HLA-C alleles of our study subjects. Since the HLA-C alleles of our study subjects was not defined and Robbins *et al* did not exclude the HLA-Cw7 allele as the presenting MHC class I molecule it is possible that these epitopes are restricted by HLA-Cw7 instead of HLA-B*0801 [544]. This might also explain why only one study subject responded to the NP_{30-38} epitope (data not shown). Alternatively, differences in HLA make up

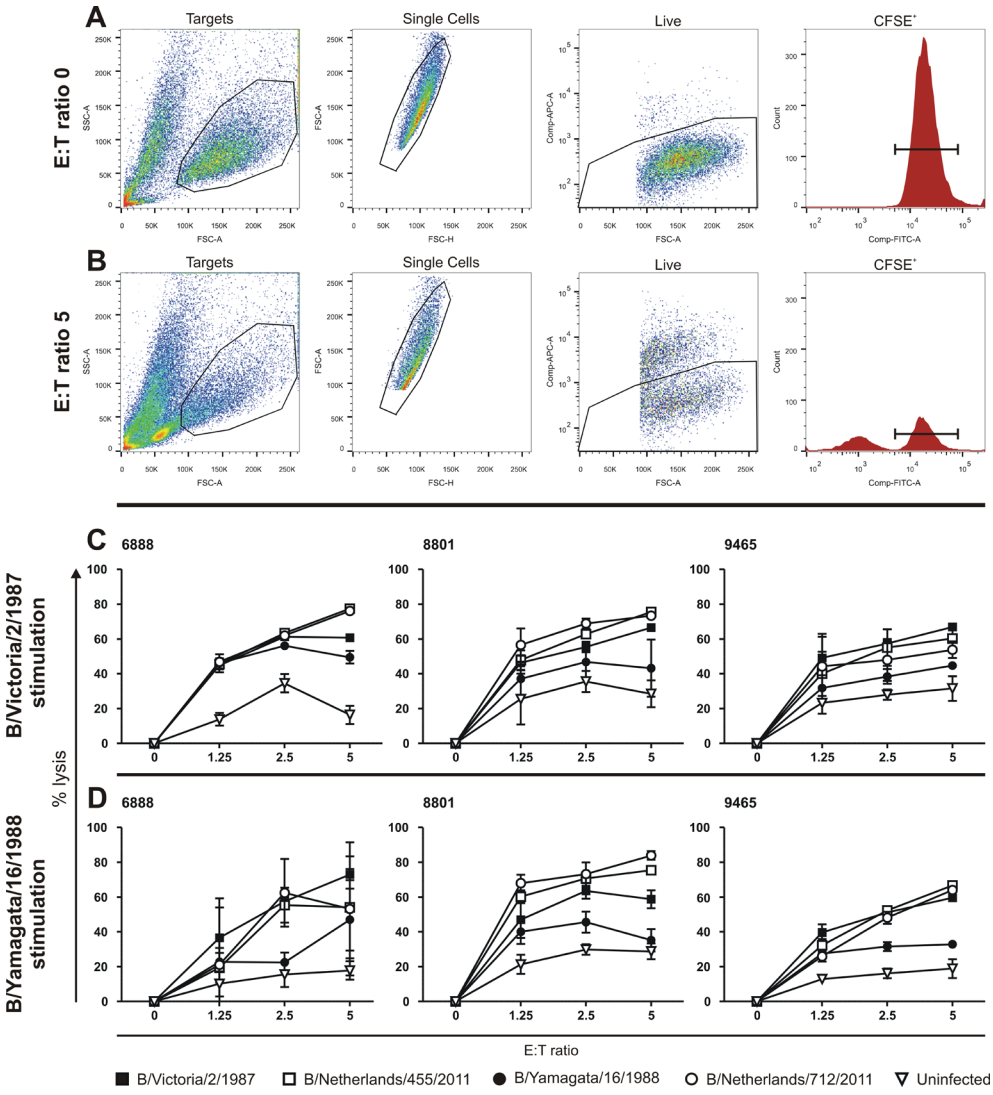

Figure 4 Cross-reactivity of virus-specific polyclonal CD8$^+$ T cells assessed by lytic activity.

B/Victoria/2/1987 (C) and B/Yamagata/16/1988 (D) virus-specific polyclonal CD8$^+$ T cells from study subjects 6888, 8801 and 9465 were isolated after stimulation of PBMCs with the respective virus. Lytic activity against HLA-matched CFSE-labeled BLCLs infected with B/Victoria/2/1987 (solid squares), B/Netherlands/455/2011 (open squares), B/Yamagata/16/1988 (solid circles) and B/Netherlands/712/2011 (open circles) was assessed. Uninfected BLCLs were used as negative controls (open triangles). Experiments were performed in triplicate. The error bars represent standard deviations of the triplicates. An example of the gating strategies used for determining the extent of specific lysis are shown for E:T ratio 0 (A) and E:T ratio 5 (B) using FlowJo software.

may have influenced immunodominance patterns [313]. Thus results obtained with epitope prediction algorithms should be interpreted with caution, which is in agreement with previous studies [556]. Therefore, viral vectors expressing a single influenza B viral protein and/or overlapping peptide pools are considered more useful than *in silico* predictions for the identification of $CD8^+$ T cell epitopes and establishing their immunodominance patterns.

The extent of cross-reactivity of influenza B virus-specific polyclonal $CD8^+$ T cells was tested with two independent assays, the IFN-γ ELISpot and CTL assay. In both assays, B/Vic and B/Yam-specific $CD8^+$ T cell populations displayed a high degree of cross-reactivity with drifted intra-lineage variants and across lineages, confirming that influenza B viruses indeed contain cross-reactive CTL epitopes. Differences in magnitude of the influenza B virus-specific $CD8^+$ T cell responses between study subjects may reflect differences in HLA class I makeup [313] and/or differences in the history of influenza infections.
Most likely, also subjects with alternative HLA-alleles will mount cross-reactive $CD8^+$ T cell responses, considering the high level of sequence identity. Thus in theory, infection with any influenza B virus will induce a cross-reactive influenza B virus-specific $CD8^+$ T cell response. These cross-reactive $CD8^+$ T cells may afford some degree of protection against a subsequent infection with an antigenically distinct influenza B virus, to which antibodies induced by previous infection will not be protective. Indeed it was demonstrated during the pandemic of 2009, that in the absence of virus neutralizing antibodies the frequency of influenza A virus-specific $CD8^+$ T cells correlated with a favorable disease outcome [344].

To address the problem of two co-circulating antigenically distinct influenza B lineages, quadrivalent seasonal influenza vaccines have become available that contain components of both influenza B lineages [464, 465, 533]. Since quadrivalent vaccines elicit antibody responses against viruses of both lineages [466-469], they eliminate the risk that the incorrect B lineage is selected for inclusion in the vaccine. However, unforeseen antigenic drift within either influenza B lineage may affect vaccine effectiveness, although not as dramatic as a mismatch of lineage [457].
Vaccines that would induce cross-reactive T cell mediated immunity may offer another layer of protective immunity that is less sensitive to antigenic drift or circulation of an opposing lineage of influenza B virus. In particular the use of live attenuated influenza vaccines have been shown to induce virus-specific $CD8^+$ T cells, in contrast to inactivated vaccines [495, 560]. Of interest, the viral proteins of live attenuated vaccines currently in use also display a high degree of sequence identity with recent circulating strains (93-100% for B/Ann Arbor/1/66, data not shown), which supports the notion that these vaccines may also induce $CD8^+$ T

cell responses that cross-react with epidemic strains of the opposing lineage.

In conclusion, the present study shows for the first time, to the best of our knowledge, that human influenza B virus-specific $CD8^+$ T cells are highly cross-reactive with influenza B viruses of the opposing lineage. Although quadrivalent influenza vaccines will be more commonly used in the near future, the induction of cross-reactive virus-specific T cell responses may be a promising approach to broaden the protective efficacy of influenza vaccines, against both influenza A and B viruses. The induction of virus-specific CTL responses may be achieved with live attenuated influenza vaccines, especially in children [560]. However, the administration of live attenuated influenza vaccines is restricted for certain high-risk groups [464, 500]. The induction of virus-specific $CD8^+$ T cell responses may also be achieved for example by the use of specific adjuvants [561-563] or novel antigen delivery systems [509, 510, 512, 513, 564].

Supporting Material for

Influenza B virus-specific CD8$^+$ T-lymphocytes strongly cross-react with viruses of the opposing influenza B lineage

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Table S1 Previously published HA accession numbers of influenza B viruses used for phylogeny from the NCBI and GISAID influenza database

Yamagata lineage	NCBI*	GISAID†	Victoria lineage	NCBI*	GISAID†
B/Hubei-Wujiagang/158/2009	CY115383		B/Singapore/616/2008		EPI_ISL28576
B/Florida/4/2006	KF009552		B/Philippines/1617/2010		EPI_ISL83789
B/Bangladesh/3333/2007	CY115255		B/Brisbane/33/2008	FJ766843	
B/Wisconsin/1/2010	CY115183		B/Bangladesh/5945/2009	CY115359	
B/Texas/6/2011	KC813979		B/HongKong/259/2010	CY115191	
B/Stockholm/12/2011		EPI_ISL90776	B/Nevada/3/2011	KC813804	
B/Massachusetts/2/2012	KC892118		B/Texas/2/2013	KF216858	
B/Estonia/55669/2011		EPI_ISL90663	B/Hongkong/330/2001	CY018709	
B/Malaysia/412/2012		EPI_ISL128746	B/Townsville/2/2008	CY153146	
B/Wellington/3/20121		EPI_ISL134527	B/Hubei/songzi/51/2008		EPI_ISL28271
B/Brisbane/36/2012		EPI_ISL134485	B/Bangladesh/4008/2008		EPI_ISL28247
B/Sichuan-Anyue/139/2011		EPI_ISL90803	B/Malaysia/2506/2004	CY040449	
B/Brisbane/3/2007	CY155898		B/Sydney/12/2008	CY153026	
B/Jiangsu/10/2003	CY033844		B/Brisbane/32/2002	CY018701	
B/Christchurch/2/2007	CY155890		B/Hongkong/1434/2002	CY018685	
B/Johannesburg/1197/2007		EPI_ISL20652	B/Hongkong/1351/2002	CY018861	
B/Jilin/20/2003	CY033828		B/Beijing/1/87	X53098	
B/Johannesburg/5/99	CY018613				
B/Harbin/7/94	CY040441				
B/Georgia/2/98	CY018405				
B/Arizona/1/2012	KC892020				
B/Iowa/1/2012	KC892010				
B/Utah/1/2012	KC891940				
B/Utah/2/2012	JX827494				
B/Washington/1/2012	CY115183				
B/NewMexico/04/2012	KF216453				
B/Yokohama/82/2012		EPI_ISL133555			
B/Yamanashi/166/98					
B/Shizuoka/15/01	CY019515				
B/Panama/45/90	CY018349				

* National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genomes/FLU)
† Global Initiative on Sharing Avian Influenza Data (GISAID; http://gisaid.org)

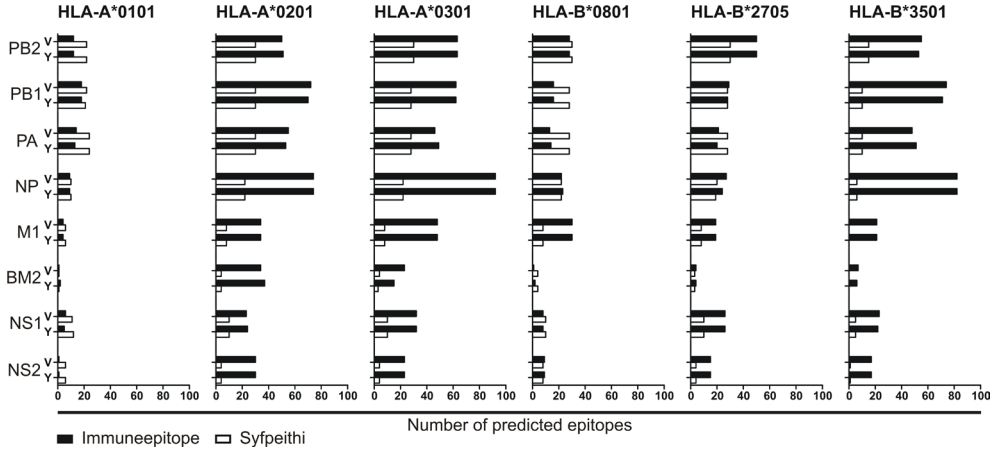

Figure S1 Absolute number of putative epitopes predicted by Sypeithi or Immuneepitope.
Syfpeithi (white bars) and Immuneepitope (black bars) algorithms were used to predict putative epitopes in B/Victoria/2/1987 (V) and B/Yamagata/16/1988 (Y) viruses for the indicated viral proteins (X-axis). Number of epitopes predicted by either program is indicated on the Y-axis). Epitopes predicted by both algorithms are shown in figure 2 and epitope sequences are available on request.

CHAPTER 3:

Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus

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Abstract

In February 2013 zoonotic transmission of a novel influenza A virus of the H7N9 subtype was reported in China. Although at present no sustained human-to-human transmission has been reported, a pandemic outbreak of this H7N9 virus is feared. Since neutralizing antibodies to the hemagglutinin (HA) globular head domain of this virus are virtually absent in the human population, there is interest in identifying other correlates of protection, such as cross-reactive $CD8^+$ T cells (cytotoxic T lymphocytes (CTLs)) elicited during seasonal influenza A virus infections. These virus-specific $CD8^+$ T cells are known to recognize conserved internal proteins of influenza A viruses predominantly, but it is unknown to what extent they cross-react with the newly emerging H7N9 virus. Here, we assessed the cross-reactivity of seasonal H3N2, H1N1 and pandemic H1N1 influenza A virus-specific polyclonal $CD8^+$ T cells, obtained from HLA-typed study subjects, with the novel H7N9 virus. The cross-reactivity of $CD8^+$ T cells to H7N9 variants of known influenza A epitopes and H7N9 virus infected cells was determined by their IFN-γ response and lytic activity. It was concluded that, apart from recognition of individual H7N9 variant epitopes, $CD8^+$ T cells to seasonal influenza viruses display considerable cross-reactivity with the novel H7N9 virus. The presence of these cross-reactive $CD8^+$ T cells may afford some protection against infection with this new virus.

Introduction

Influenza viruses are an important cause of respiratory tract infections. Occasionally, animal influenza viruses cross the species barrier and infect humans after zoonotic transmission. In the past two decades several avian influenza A viruses have infected humans, like those of the H9N2 subtype [109], the H7N7 subtype [127, 128] and the H5N1 subtype [116-120, 565]. In 2009, H1N1 influenza A viruses of swine origin (H1N1pdm09) caused a pandemic outbreak and these viruses continue to circulate in the human population [84].

In February 2013 the first human cases of infection with a novel avian influenza A virus of the H7N9 subtype were reported in China. To date (September 2013), 135 laboratory confirmed cases have been reported of which 44 had a fatal outcome [566]. Especially older male individuals seem to be at risk to develop severe disease upon infection [567-570]. Most hospitalized patients developed severe viral pneumonia and acute respiratory distress syndrome (ARDS) [137-140].

Influenza A viruses with hemagglutinin and neuraminidase of the subtypes H7 and N9 respectively, circulate in wild bird species [571, 572]. The newly emerged H7N9 virus is most likely the result of multiple reassortment events of at least three avian viruses [138, 142, 143]. Although the H7N9 virus has been classified as a low pathogenic virus based on the intravenous pathogenicity index (IVPI) in chickens and the absence of a multi-basic cleavage site in the hemagglutinin (HA), it is quite pathogenic in humans [138]. This virus also replicates efficiently in the airways of other mammalian species, including mice, ferrets and cynomolgus macaques [145, 146]. It is more pathogenic than seasonal influenza A H3N2 (sH3N2) viruses or pandemic 2009 H1N1 (pH1N1) viruses and after intratracheal inoculation causes fatal disease in ferrets [573]. The high pathogenicity in mammals correlates with the presence of known pathogenicity markers. Several human isolates of the H7N9 virus contain the E627K substitution in PB2, which allows avian influenza viruses to replicate at lower temperatures [574]. A deletion of 5 amino acids in the neuraminidase (NA) of H7N9 virus is associated with enhanced virus replication [138]. The presence of the Q226L substitution in the HA [138, 144] is associated with binding to alpha (2,6)-linked sialic acids found in the human upper respiratory tract [145] and has been associated with airborne transmission of avian H5N1 virus in ferrets [88]. In case of the novel H7N9 virus, only limited transmission between ferrets was observed [145-148]. Acquisition of gene segments from human influenza A viruses by the avian influenza H7N9 virus through genetic reassortment may lead to further adaptation to humans [81, 83, 84, 86, 87, 155, 528]. The detection of a H7N9 patient that was co-infected with a sH3N2 virus underscores this possible scenario [154]. Although at present no sustained human-to-human transmission of the H7N9 virus has been reported [150], the pandemic potential of H7N9 virus should be considered seriously. Especially since virus neutralizing antibodies directed to the HA globular head domain of this virus are virtually absent in the human population

[139], though low concentrations of stalk-region specific antibodies might be present [244, 251].

On the other hand, virus-specific $CD8^+$ T cells (cytotoxic T lymphocytes (CTL)), induced after infection with seasonal influenza A viruses, are mainly directed to the conserved internal proteins of influenza A viruses [311, 312, 314-317, 410, 450, 528, 534, 535]. The presence of these cross-reactive $CD8^+$ T cells may afford a certain degree of heterosubtypic immunity against infection with novel H7N9 viruses. Using various combinations of influenza A virus subtypes for primary and secondary infection, this type of immunity and the contribution of virus-specific $CD8^+$ T cells was demonstrated in various animal models [337-339, 538-540]. Evidence for heterosubtypic immunity and the role of $CD8^+$ T cells in humans is limited [344-347], though the presence of $CD8^+$ T cells cross-reactive with avian H5N1 and swine-origin triple reassortant A H3N2 (vH3N2) viruses has been demonstrated [316, 317, 535, 541]. It is unknown to what extent $CD8^+$ T cells elicited by a seasonal or 2009 pH1N1 influenza A virus infection cross-react with the novel H7N9 virus. Here we show that polyclonal $CD8^+$ T cell populations specific for seasonal H1N1 (sH1N1), sH3N2 or pH1N1 virus cross-react with the H7N9 virus by determining their interferon γ (IFN-γ) response upon *in vitro* stimulation with the novel H7N9 virus and their lytic activity towards H7N9 virus infected human leukocyte antigen (HLA)-matched target cells. The pre-existing cross-reactive $CD8^+$ T cells may afford some level of protection and may reduce morbidity and mortality caused by infections with the novel H7N9 virus.

Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from 6 HLA-typed healthy blood donors (35-50 years of age), between 2008 and 2013 (Sanquin Bloodbank, Rotterdam, the Netherlands). Lymphoprep (Axis-Shield PoC, Oslo, Norway) gradient centrifugation was used to isolate PBMCs which were subsequently cryopreserved at -135°C. Donors were selected based on their HLA class I alleles for which functionally confirmed influenza A HLA class I epitopes have been identified and had the following HLA-haplotypes: subject 1 and 2: HLA-A*0101, A*0201, B*0801, B*3501; subject 3 and 4: HLA-A*0101, A*0201, B*0801, B*2705; subject 5 and 6: HLA-A*0101, A*0301, B*0801, B*3501. The use of PBMCs for scientific research was approved by the Sanquin Bloodbank after informed consent was obtained from the blood donors.

Peptides

Amino acid sequences of confirmed influenza A HLA class I epitopes were aligned with their H7N9 analogues from human isolates between February 2013 and April 22th 2013 (Table 1). Sequences were obtained from the influenza resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=-

database). In addition, conservation of these epitope sequences in the prototype viruses used in the present study: sH3N2 (A/Netherlands/348/07), sH1N1 (A/Netherlands/26/07) and pH1N1 (A/Netherlands/602/09) was determined (Table 1). The H7N9 variant epitopes, of which the HLA restriction was compatible with the HLA-type of the study subjects, were ordered as synthetic immunograde peptides (>85% purity) (Eurogentec, Seraing, Belgium).

Table 1 Variant amino acid sequences of known CD8⁺ T cell epitopes in the influenza A H7N9 virus [a]

		Amino acid sequence [b]					
HLA	**Epitope**	**Influenza A virus**	**sH3N2**	**sH1N1**	**pH1N1**	**H7N9**	**Refs**
A*3	M1 13-21	SIIPSGPLK	--V------	--V------	---------	---------	[575]
	M1 27-35	RLEDVFAGK	---------	---S-----	---------	---------	[576]
A*0201	M1 58-66	GILGFVFTL	---------	---------	---------	---------	[312, 577]
	M1 59-68	ILGFVFTLTV	----------	----------	----------	----------	[577]
B*35	M1 128-135	ASCMGLIY	--------	--------	--------	--------	[578]
B*44	M2 7-15	VETPIRNEW	---------	---------	----T-S--	----T-TG-	[315]
A*0201	NA 213-221	CVNGSCFTV	INGTCTVVM	--------I	---------	VCPVVFTDG	[579]
A*01	NP 44-52	CTELKLSDY	--------H	------N--	---------	--------N	[580]
A*6801	NP 91-99	KTGGPIYKR	-------R-	---------	-------R-	-------R-	[581]
B*1402	NP 146-154	TTYQRTRAL	A--------	---------	A--------	A--------	[582]
B*2705	NP 174-184	RRSGAAGAAVK	----------	----------	----------	----------	[541]
A*3	NP 188-198	TMVMELVRMIK	------I--V-	---L--I----	-IA---I----	------I----	[575]
A*03	NP 265-273	ILRGSVAHK	---------	---------	---------	---------	[580]
B*44	NP 338-346	FEDLRVLSF	-----L---	------S--	------S--	------S--	[583]
B*3701	NP 339-347	EDLRVLSFI	----L----	-----S---	-----S---	-----S---	[584, 585]
B*44	NP 379-387	LELRSRYWA	-----G---	---------	---------	---------	[583]
B*0801	NP 380-388	ELRSRYWAI	----G----	---------	---------	---------	[586]
B*2702	NP 381-388	LRSRYWAI	---G----	--------	--------	--------	[587]
B*2705	NP 383-391	SRYWAIRTR	-G-------	---------	---------	---------	[588]
B*35	NP418-426	LPFEKSTVM	-------I-	---D-A-I-	----RA---	----RA-I-	[441]
A*0201	NS1 122-130	AIMDKNIIL	---E---M-	---------	---E---V-	--V----T-	[589]
	NS1 123-132	IMDKNIILKA	--E---M---	----------	--E---V---	-V----T---	[589]
B*44	NS1 158-166	GEISPLPSL	--------F	--------F	---------	---------	[583]
A*01	PB1 591-599	VSDGGPNLY	---------	---------	---------	---------	[580]

[a] The A/Anhui/1/13 (H7N9) sequence was unavailable at the time of ordering the peptides. All epitopes, except LPFEKSTVM (H7N9 LPFERARIM), were conserved between the H7N9 viruses present in the database at April 22th and the A/Anhui/1/13 virus used in this study.

[b] Peptides used in the present study are shaded and were selected based on variation in the H7N9 sequence and correspondence to the HLA alleles of the study subjects. Synthetic immunograde peptides were ordered with >85% purity. The dashes indicate identity with the amino acids in the influenza A virus sequences.

Viruses

Influenza virus A/Anhui/1/2013 (H7N9) was isolated from a fatal human case (Anhui province, People's Republic of China) and was kindly provided through the WHO Pandemic Influenza Preparedness (PIP) framework and subsequently passaged once in Madin Darby Canine Kidney (MDCK) cells. Prototypic seasonal influenza A viruses A/Netherlands/348/07 (sH3N2), A/Netherlands/26/07 (sH1N1) and A/Netherlands/602/09 (pH1N1) were propagated in MDCK cells. Culture supernatants were clarified by low speed centrifugation and subsequently purified by ultra centrifugation through a sucrose gradient. Their infectious virus titers were determined as described previously [545].

Amino acid sequence identity

Amino acid sequence identity of the viral proteins of influenza viruses A/Anhui/1/2013 (H7N9) and the prototype sH3N2, sH1N1 and pH1N1 was determined using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 2). The consensus sequence of A/Anhui/1/2013 was obtained from the GISAID database (http://platform.gisaid.org) and the consensus sequence of the influenza virus A/Anhui/1/2013 preparation used in the present study was confirmed by sequence analysis [147].

Table 2 Percent amino acid sequence identity with A/Anhui/1/2013 (H7N9)

	% Identity		
Gene segment	**sH3N2**	**sH1N1**	**pH1N1**
PB2	94	94	97
PB1	97	95	96
PA	94	95	96
HA	47	41	41
NP	91	92	93
NA	45	43	45
M1	91	91	92
M2	82	78	89
NS1	76	80	78
NS2	93	90	88

In vitro expansion of influenza A virus-specific $CD8^+$ T cells

PBMCs obtained from HLA-typed study subjects were stimulated with sH3N2, sH1N1 and pH1N1 viruses at a multiplicity of infection (MOI) of three as described previously [313]. Eight days after stimulation, polyclonal $CD8^+$ T cells were isolated from the expanded PBMC cultures by means of $CD8^+$ magnetically activated cell sorting (MACS) bead sorting according to the manufacturer's recommendation (Miltenyi Biotec, Bergisch Gladbach, Germany) and subsequently used as effector cells in IFN-γ ELISpot and lytic assays (see below).

Target cells

HLA-matched B-Lymphoblastoid cell lines (BLCLs) were prepared as described previously [513]. 10^6 cells were incubated with or without 100µM peptide for 16 hours at 37°C and subsequently washed and resuspended in RPMI1640 medium (Lonza, Basel, Switzerland) containing antibiotics and 10% fetal bovine serum (Sigma-Aldrich, Zwijndrecht, The Netherlands) (R10F medium). Virus-infected target cells were prepared by inoculating BLCLs at a MOI of three with sH3N2, sH1N1, pH1N1 or H7N9 virus. After one hour, cells were washed and resuspended in R10F medium and cultured for 16-18 hours at 37°C before being used for the stimulation of T cells or as target cells.

IFN-γ ELISpot assay

IFN-γ responses of *in vitro*-expanded polyclonal $CD8^+$ T cells were determined by enzyme-linked immunospot (ELISpot) assays, which were performed according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden). In brief, 10,000 or 5,000 *in vitro* expanded polyclonal $CD8^+$ T cells were used as effector cells and incubated for 16-18 hours with 30,000 peptide-loaded, virus-infected or untreated HLA class I-matched target cells, in triplicate. The average number of spots was determined using an ELISpot reader and image analysis software (Aelvis, Sanquin Reagents, Amsterdam, The Netherlands).

CTL assay

The lytic capacity of the *in vitro* expanded polyclonal $CD8^+$ T cells was determined using a CTL assay with Carboxyfluorescein Succinimidyl Ester (CFSE) labeled target cells. In brief, 5.10^6 cells of HLA class I-matched BLCLs were incubated with 0,6µM CFSE (Sigma-Aldrich, Zwijndrecht, The Netherlands) for five minutes at 37°C. Subsequently these cells were inoculated with sH3N2, sH1N1, pH1N1 or H7N9 virus at a MOI of three for 16-18 hours. The infected and CFSE-labeled BLCL target cells were co-cultured with the *in vitro* expanded polyclonal $CD8^+$ T cells effector cells in effector:target (E:T) ratios of 5, 2.5 and 1.25. After a three hour incubation period cells were fixed using Cytofix/Cytoperm (BD Biosciences, Breda, The Netherlands) and lysis in the target cell population was determined by flow cytometry using BD FACSDiva software (Becton Dickinson B.V., Breda, The Netherlands). Experiments were performed in triplicate.

Statistical analysis

The data were analyzed using an independent T test and differences were considered significant at $p<0.05$.

Results

Comparison of amino acid sequences of CD8⁺ T cell epitopes

Amino acid sequences of 24 confirmed influenza A virus HLA class I epitopes were compared with their influenza A H7N9 virus analogues. As shown in Table 1 most epitopes (>50%) were fully conserved in H7N9 viruses. Based on these results, four variant H7N9 epitopes that were conserved in our prototypic sH3N2, sH1N1 and/or pH1N1 viruses (Table 1) and were compatible with the HLA-type of the study subjects under investigation were further tested for cross-recognition in the ELISpot assay. All epitopes, except $NP_{418-426}$, were conserved among H7N9 viruses available in the influenza resource database (April 22th 2013) and the A/Anhui/1/13 (H7N9) virus used in this study.

Cross-recognition of influenza A(H7N9) analogues of known influenza A HLA class I epitopes

In vitro expanded CD8⁺ T cell preparations specific for sH3N2, sH1N1 and pH1N1 influenza viruses were tested for their cross-reactivity with the selected H7N9 variant epitopes listed in table 1 using peptide-loaded HLA-matched BLCLs.

Virus-specific CD8⁺ T cell obtained from study subjects 1 and 2 (HLA-A*0101, A*0201, B*0801, B*3501) displayed strong reactivity with the homologous epitopes, except for epitope $NS_{123-132}$ (IMDKNIILKA) (Fig. 1A and B). The H7N9 variant of the $NP_{418-426}$ (LPFERATIM) epitope was recognized by sH3N2 specific CD8⁺ T cells derived from subject 1, although the IFN-γ response was lower than the response to the homologous epitope (LPFEKSTIM) (Fig. 1A). None of the other H7N9 variant epitopes were recognized by virus specific CD8⁺ T cells of these HLA-A*0101, A*0201, B*0801, B*3501 study subjects. Virus-specific CD8⁺ T cells obtained from study subjects 3 and 4 (HLA-A*0101, A*0201, B*0801, B*2705) displayed a minor response to homologous epitopes $NS_{122-130}$ and $NS_{123-132}$ (Fig. 1C and D). This is in agreement with the subdominant nature of the response to these epitopes in these subjects (data not shown). CD8⁺ T cells from both subjects did not display any response to the H7N9 variant of the $NS_{122-130}$ and $NS_{123-132}$ epitopes (Fig. 1C and D). Although CD8⁺ T cells of these two subjects displayed reactivity with homologous NP_{44-52} epitope, they did not respond to the H7N9 variant of this epitope (CTELKLSDN). Virus-specific CD8⁺ T cells from study subjects 5 and 6 (HLA-A*0101, A*0301, B*0801, B*3501) displayed a strong response to the homologous sH3N2 variant of the $NP_{418-426}$ epitope (Fig. 1E and F). Some minor cross-reactivity with the H7N9 variant (LPFERATIM) was observed with CD8⁺ T cells derived from subject 5 (Fig. 1E). As for the other subjects, no cross reactivity was observed with the H7N9 variant of NP_{44-52} (CTELKLSDN) epitope with CD8⁺ T cells obtained from subject 5.

Overall, the extent of cross-reactivity of influenza virus-specific CD8⁺ T cells against individual H7N9 variant epitopes was low and dependent on study subjects and peptides tested.

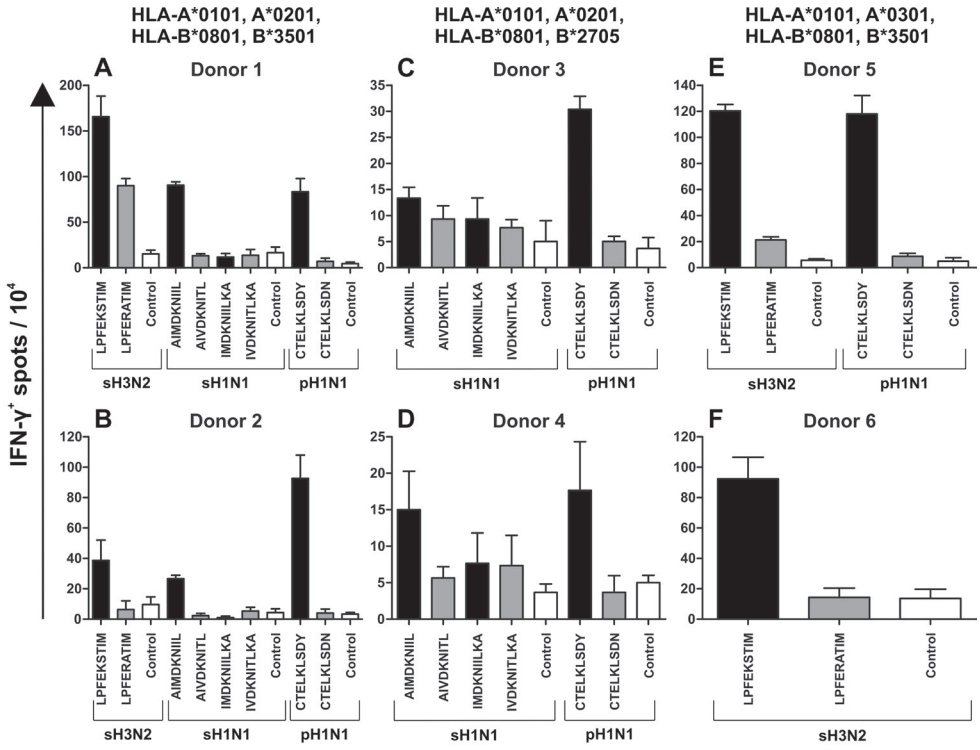

Figure 1 Epitope-specific IFN-γ production by seasonal influenza virus-specific CD8⁺ T cells after stimulation with peptide-loaded BLCLs.

Polyclonal CD8⁺ T cells were isolated from PBMCs *in vitro* stimulated with sH3N2, sH1N1 or pH1N1 as indicated. No pH1N1 *in vitro* stimulation was performed for subject 6 since these PBMCs were isolated in 2008 prior to the 2009 pandemic outbreak. Polyclonal CD8⁺ T cells were subsequently stimulated with peptide-loaded and untreated HLA class I matched BLCLs. Stimulation with homologous peptides is indicated by black bars, with H7N9 variant peptides by grey bars and control cells without peptide by white bars. The number of IFN-γ producing cells per 10,000 polyclonal CD8⁺ T cells was determined by ELISpot assay. The results represent the average of triplicate wells. Peptides were selected based on the variation in the H7N9 sequence and their compatibility with the HLA-haplotypes of our study subjects. The error bars indicate standard deviations of results from the triplicate wells.

CD8⁺ T cells cross-react with influenza A H7N9 virus infected cells

Since more than 50% of previously identified influenza HLA class I epitopes were present in the H7N9 virus we wished to compare the overall amino acid sequence identity between the H7N9 virus and the prototypic sH3N2, sH1N1 and pH1N1 viruses used in the present study. BLAST analysis revealed that the sequence identity of most viral proteins, was high (>76%), except for the hemagglutinin and neuraminidase (Table 2).

Since the sequence identity between seasonal influenza viruses used in this study and H7N9 virus is high, we wished to determine the cross-reactivity of polyclonal CD8⁺ T cells specific for sH3N2, sH1N1 or pH1N1 viruses with H7N9 virus. To this end, *in vitro* expanded seasonal influenza virus-specific polyclonal CD8⁺ T cells were

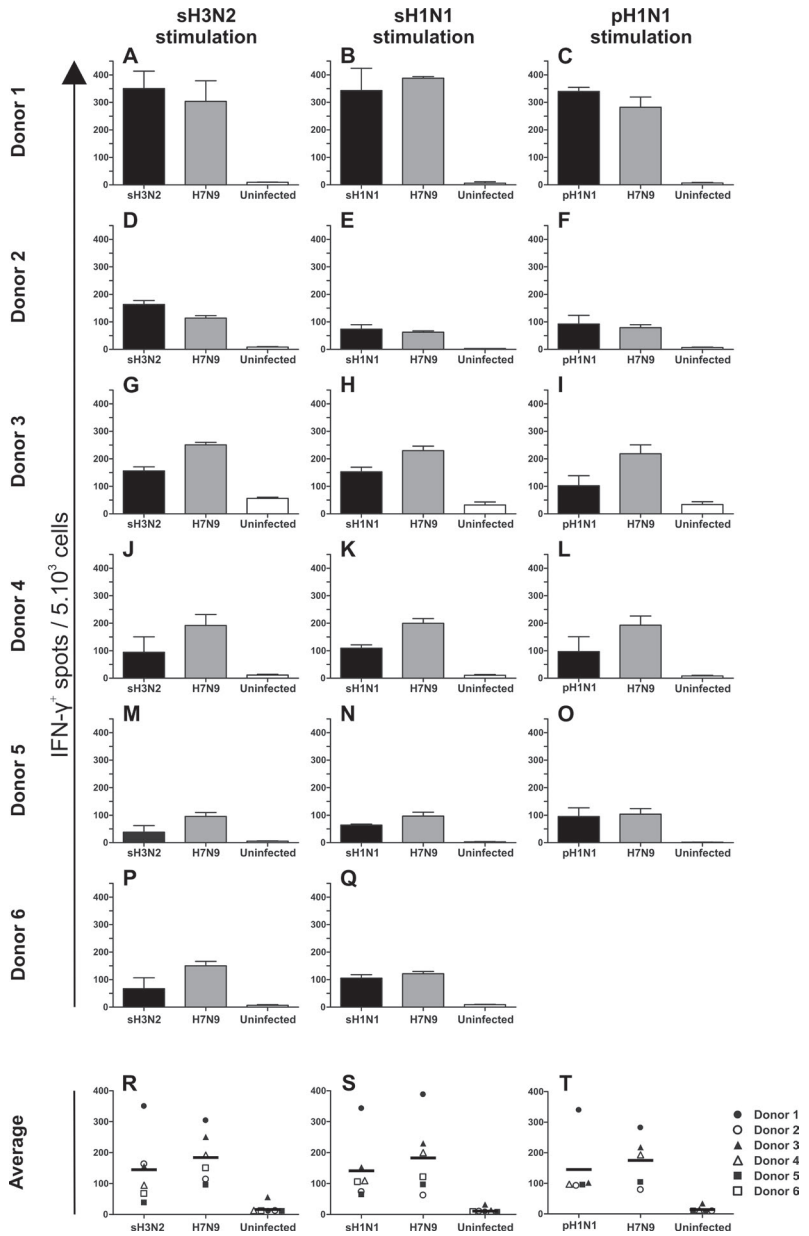

Figure 2 Virus-specific IFN-γ production by polyclonal CD8⁺ T cells after stimulation with BLCLs infected with homologous or H7N9 virus.

Seasonal influenza virus-specific polyclonal CD8⁺ T cells were isolated from PBMCs stimulated with sH3N2 (A, D, G, J, M, P), sH1N1 (B, E, H, K, N, Q) or pH1N1 (C, F, I, L, O). PBMCs of subject 6 were not stimulated *in vitro* with pH1N1 since they were isolated prior to the pH1N1 outbreak. The CD8⁺ T cells were subsequently co-cultured with BLCLs infected with homologous virus (sH3N2, sH1N1 or pH1N1) (black bars) or the heterologous novel H7N9 virus (grey bars). The number of IFN-γ producing cells per 5,000 polyclonal CD8⁺ T cells was determined by ELISpot assay. Uninfected BLCLs were used as negative controls (white bars). Experiments were performed in triplicate. The symbols in figure R, S and T represent the average of triplicates for each individual subject and the horizontal bars represent the average response of all study subjects combined.

stimulated with HLA class-I matched BLCLs infected with the homologous seasonal influenza A virus or H7N9 virus (A/Anhui/1/2013). The number of IFN-γ-producing cells per 5,000 $CD8^+$ T cells was determined in an IFN-γ ELISpot assay (Figure 2).
Study subject 1 showed a high response to homologous seasonal influenza viruses (sH3N2, sH1N1 and pH1N1) but also after stimulation with H7N9 virus infected cells (Figure 2A, B and C). Although the frequency of seasonal influenza virus specific $CD8^+$ T cells derived from subject 2 was lower than that of subject 1, these T cells also cross-reacted with H7N9 virus infected cells (Figure 2D, E and F).Subjects 3 and 4 responded both to the homologous viruses and H7N9 virus (Figure 2G, H, I, J, K and L). Subjects 5 and 6 (which lack the HLA-A*0201 allele) showed the lowest response to stimulation with homologous viruses. However, virus specific $CD8^+$ T cells of these two subjects also displayed cross-reactivity with H7N9 virus (Figure 2M, N, O, P and Q).
Thus, although the frequency of virus-specific IFN-γ producing T cells varied between the study subjects, these cells cross-reacted with the H7N9 virus. This was independent of the sH3N2, sH1N1 or pH1N1 virus used for the *in vitro* expansion of the polyclonal $CD8^+$ T cells (Figure 2). The average number of spots tended to be higher after re-stimulation with H7N9 virus compared to re-stimulations with the homologous viruses, although this difference was not statistically significant (Figure 2R, S and T).

Cross-recognition of $CD8^+$ T cells with influenza A H7N9 virus assessed by lytic activity

Based on the IFN-γ ELISpot results, we selected high-responding study subjects from each HLA-group to test the lytic capacity of the $CD8^+$ T cells against HLA class-I matched BLCLs infected with the homologous or H7N9 virus. To this end, polyclonal $CD8^+$ T cells derived from sH3N2, sH1N1 or pH1N1 virus stimulated PBMC cultures from study subjects 1, 3 and 5 were incubated with CFSE-labeled BLCLs infected with the sH3N2, sH1N1, pH1N1 or H7N9 virus.
$CD8^+$ T cells from subject 1 obtained after sH3N2, sH1N1 and pH1N1 virus stimulation not only displayed lytic activity to the respective homologous viruses, but also displayed similar or even stronger lytic activity to H7N9 virus infected cells as was observed for sH1N1 virus-specific $CD8^+$ T cells (Figure 3A, B and C). A similar trend was observed for virus-specific $CD8^+$ T cells obtained from subject 3. Again, the lytic activity to H7N9 virus infected cells exceeded that of cells infected with the homologous viruses to various extents (Figure 3D, E and F). Virus-specific $CD8^+$ T cells of subject 5 displayed minor lytic activity to target cells infected with the respective homologous viruses. Again the lytic activity to target cells infected with H7N9 virus exceeded that of cells infected with the homologous viruses and uninfected control cells (Figure 3G, H and I). The background lytic activity of T cells derived from subjects 1 and 5 was high, which may be related to bystander proliferation of EBV-specific T cells.

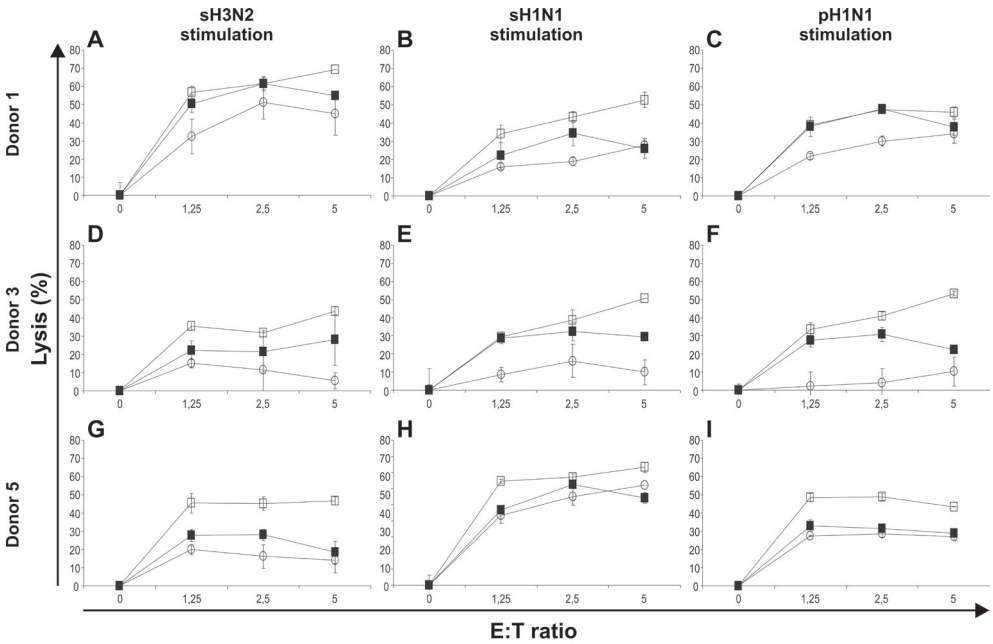

Figure 3 Lytic activity of virus-specific polyclonal CD8$^+$ T cells against BLCLs infected with the homologous or H7N9 virus.

Seasonal influenza virus-specific polyclonal CD8$^+$ T cells from study subject 1, 3 and 5 were isolated after stimulation with sH3N2 (A, D, G), sH1N1 (B, E, H) or pH1N1 (C, F, I) virus, as indicated. Lytic activity against CFSE labled BLCLs infected with the homologous virus (sH3N2, sH1N1 or pH1N1) (closed squares) or the heterologous novel H7N9 virus (open squares) was assessed even as lytic background activity against uninfected cells (open circles). Experiments were performed in triplicate. The error bars indicate standard deviations for the triplicates.

Discussion

Here we assessed the cross-reactivity of seasonal influenza A virus-specific CD8$^+$ T cells with the newly emerging H7N9 virus. This study showed that a significant proportion of the polyclonal CD8$^+$ T cells specific for sH3N2 (A/Netherlands/384/07), sH1N1 (A/Netherlands/26/07) and pH1N1 (A/Netherlands/602/09) are cross-react with the novel H7N9 virus (A/Anhui/1/2013).

Comparison of epitope sequences revealed that the majority of the currently known HLA class I epitopes is conserved in the novel H7N9 viruses. Several studies have shown that the conservation of these HLA class I epitopes is responsible for cross-reactivity of influenza A virus-specific CD8$^+$ T cells with influenza A viruses of another subtype [311, 314, 316, 317, 535]. However, variation in some of the known epitopes was observed. We demonstrated that there is very little cross-reactivity of seasonal influenza A virus-specific CD8$^+$ T cells with four individual H7N9 variant epitopes, although CD8$^+$ T cells of subject 1 displayed some cross-reactivity with the H7N9 $NP_{418-426}$ (HLA-B*35 restricted) epitope (Figure 1A). The magnitude of the response to individual peptides varied between study subjects (Figure 1). These

differences may reflect differences in HLA class I make-up [313] and/or differences in the history of influenza A virus infections.
Although it has been suggested that the novel H7N9 virus is poorly immunogenic based on the *in silico* predictions of T cell epitopes in HA [590], we clearly demonstrate that the presence of most conserved HLA class I epitopes in the novel H7N9 virus contributes to the high cross-reactivity of the polyclonal $CD8^+$ T cell populations with the H7N9 virus (Figure 2 and 3). The low IFN-γ response of study subject 5 and 6 (A*0101, A*0301, B*0801, B*3501) to stimulation with the homologous seasonal influenza viruses and the H7N9 virus (Figure 2) might be attributed to the absence of the HLA-A*0201 allele, which is required for a dominant $CD8^+$ T cell response to the conserved and $M1_{58-66}$ epitope [313]. All study subjects displayed equal or greater cross-reactive responses to H7N9 virus compared to those against the homologous viruses (Figure 2 and 3), which could not be attributed to differences in infection rate (data not shown). These results correspond with previous assessments of cross-reactive $CD8^+$ T cells with avian influenza A viruses of the H5N1 subtype [316]. The strong reactivity to avian influenza A viruses might be the result of differences in antigen processing in infected cells, allowing more peptides to be liberated and presented from viral proteins of avian viruses than from those of human influenza viruses. It can be hypothesized that since these avian viruses have not circulated in the human population extensively, they did not yet have a chance to acquire mechanisms to escape from human epitope processing [417, 528, 591-595].
Although we have studied the cross-reactivity of $CD8^+$ T cells of study subjects with selected HLA-types, it is likely that individuals with other HLA-types will also possess cross-reactive $CD8^+$ T cells. The conservation of HLA class I epitopes restricted by other HLA-alleles (Table 1) and the high amino acid sequence identity between the seasonal influenza viruses and the H7N9 virus underscores this (Table 2).

Cross-reactive influenza A virus-specific $CD8^+$ T cells are found in those individuals who experienced an influenza A virus infection at least once. In contrast, a seroprevalence study indicated that a large proportion of children under the age of four years had not experienced an influenza A virus infection and therefore may not have developed virus-specific T cell responses [287]. This age group may therefore be at higher risk of developing severe disease during a pandemic outbreak than adults. This was indeed the case during the 2009 H1N1 pandemic [103] and the localized outbreaks of the H5N1 subtype [348] and the vH3N2 subtype [596, 597]. However, in case of the novel H7N9 virus mainly older (male) individuals were at risk for developing severe disease [567-570]. The reason for this discrepancy is unknown at present. It has been suggested that differences in cell-mediated immunity between different age groups are the basis for this predilection [598]. Elderly people who had experienced a H1N1 infection before 1957 were

serologically protected during the 2009 pandemic outbreak and the following years, whereas many unprotected individuals, including children, suffered from a pH1N1 infection in recent years [103, 599]. Recent influenza A virus infections in children and young adults most likely boosted their cellular immune responses, which may afford some protection to infection with viruses of novel subtypes including those of the H7N9 subtype [598]. Others have suggested that pre-existing immunity consisting of low levels of weakly heterosubtypic antibodies may result in antibody dependent enhancement (ADE) of the infection [569]. Instead of neutralizing the virus, these antibodies would enhance uptake of the virus and thus promote its replication. It cannot be excluded that other confounding factors may have contributed to the predilection of H7N9 disease for older individuals. Elderly people are more likely to suffer from underlying diseases [600] and are known to have altered T cell-immunity which is likely to influence the outcome of an influenza A infection [601, 602].

It is difficult to predict to what extent pre-existing influenza A virus-specific $CD8^+$ T cells will afford protection against novel pandemic influenza viruses. Several animal studies have shown that virus-specific $CD8^+$ T cells contribute to hetero-subtypic immunity [337-339, 538-540]. However, evidence for heterosubtypic protection by $CD8^+$ T cells in humans is sparse [343, 345]. Epidemiologic studies showed that individuals who had experienced a seasonal H1N1 infection prior to the 1957 H2N2 pandemic were partially protected [346, 347] which could be attributed to cross-reactive T cells and/or antibodies to e.g. the stalk region of HA. A similar trend was observed in isolated H5N1 infections [348]. However, recent studies performed during the 2009 H1N1 pandemic revealed better insight into the protective role of $CD8^+$ T cells during an infection with an antigenically distinct influenza virus in serologically naïve humans. It was shown that patients developed less severe illness when they had a high frequency of pre-existing virus-specific $CD8^+$ T cells before the onset of the pandemic [344]. Another study showed that infected patients developed strong and rapid cross-reactive recall T cell responses which in most cases coincided with disappearance of clinical symptoms [603].

In conclusion, we have demonstrated that cross-reactive $CD8^+$ T cells are present in the human population that can cross-react with the newly emerging H7N9 influenza virus and that may afford some protection in the absence of virus neutralizing antibodies. Cross-reactive $CD8^+$ T cells will not establish sterile immunity, they will however contribute to a more rapid clearance of the H7N9 virus infection. Immunity afforded by the presence of cross-reactive $CD8^+$ T cells may not only reduce the severity of disease caused by H7N9 virus infection, it may also contribute to reduction of virus spread in the population, since infected individuals may be infectious for a shorter period of time. Induction of cross-reactive virus-

specific T cell responses may a promising approach for the development of universal influenza vaccines that can elicit broadly protective immunity against influenza A viruses of various subtypes.

CHAPTER 4:

Human influenza A virus-specific $CD8^+$ T cell response is long-lived

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Abstract

Animal and human studies have demonstrated the importance of influenza A virus (IAV)-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) in heterosubtypic cross-protective immunity. Using peripheral blood mononuclear cells obtained intermittently from healthy HLA-typed blood donors between 1999 and 2012, we were able to demonstrate that IAV-specific CTLs are long-lived. Intercurrent IAV infections transiently increase the frequency of functionally distinct subsets of IAV-specific CTLs, in particular effector and effector memory T cells.

Annually, influenza A virus (IAV) infections cause excess mortality and morbidity in the human population. Neutralizing antibodies (nAbs) induced by IAV infection or vaccination are mainly directed to the globular head of the hemagglutinin (HA) [68]. Lifelong protection by these nAbs is hampered by continuous antigenic drift in this region of the HA molecule [68, 79] and the introduction of antigenically distinct (pandemic) IAVs into a serologically naïve population [79]. However, IAV-specific T cells induced by infection with seasonal IAV contribute to protective immunity against these novel viruses. IAV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are predominantly directed against more conserved internal proteins and are therefore cross-reactive and provide protection against antigenically distinct IAVs [344, 528, 536] . However, the longevity of the IAV-specific human CTL response is largely unknown. Studies in mice showed that CTLs induced by IAV infection are relatively long lived [540, 604]. Here, we investigate the longevity of the human IAV-specific $CD8^+$ T-cell response using uniquely biobanked samples obtained from HLA-typed healthy study subjects.

Methods

Peripheral blood mononuclear cells (PBMCs) were obtained intermittently from nine HLA-typed healthy blood donors (18-64 years of age) between 1999 and 2012 (Sanquin Bloodbank, Rotterdam, the Netherlands) and cryopreserved (Supplementary Table 1) [536]. For most time points, blood plasma was obtained and stored at -20°C. Plasma samples were used to assess if reinfections had likely occurred between 1998-2012 by virus neutralization (VN) assay [605] using 17 representative H1N1 and H3N2 IAV strains that circulated in the Netherlands in these years. The use of PBMCs and plasma for scientific research was approved by the Sanquin Bloodbank after informed consent was obtained from the blood donors. PBMCs were stimulated with influenza A/H3N2 virus Resvir-9 (a reassortant strain containing the hemagglutinin, nucleoprotein and neuraminidase of IAV A/Nanchang/933/95 and all other genes of IAV A/Puerto Rico/8/34) to assess the frequency of IAV-specific $CD8^+$ T cells by CD69 and intracellular interferon (IFN)-γ staining (ICS) as described previously and shown in figure 1A [486]. Staphylococcus enterotoxin B (SEB) (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as positive control to confirm functional integrity of the cells after thawing, a strong response was detected with PBMC of all donors (data not shown). The frequency of IAV-specific $CD8^+$ T cells was also determined by staining with HLA-peptide oligomers (dextramers, Dm) using a cocktail of R-phycoerythrin (PE)-labeled Dms for highly conserved IAV CTL epitopes corresponding to the HLA haplotypes of the blood donors (Supplementary Table 1): HLA-A*0101-$PB1_{591-599}$ (VSDGGPNLY), HLA-A*0201-$M1_{58-66}$(GILGFVFTL), HLA-A*0301-$NP_{265-273}$(ILRGSVAHK), HLA-B*0801-$NP_{380-388}$(ELRSRYWAI), HLA-B*2705-$NP_{174-184}$(RRSGAAGAAVK) (Immudex, Copenhagen, Denmark). Briefly, $2x10^6$ cells were washed extensively with phosphate

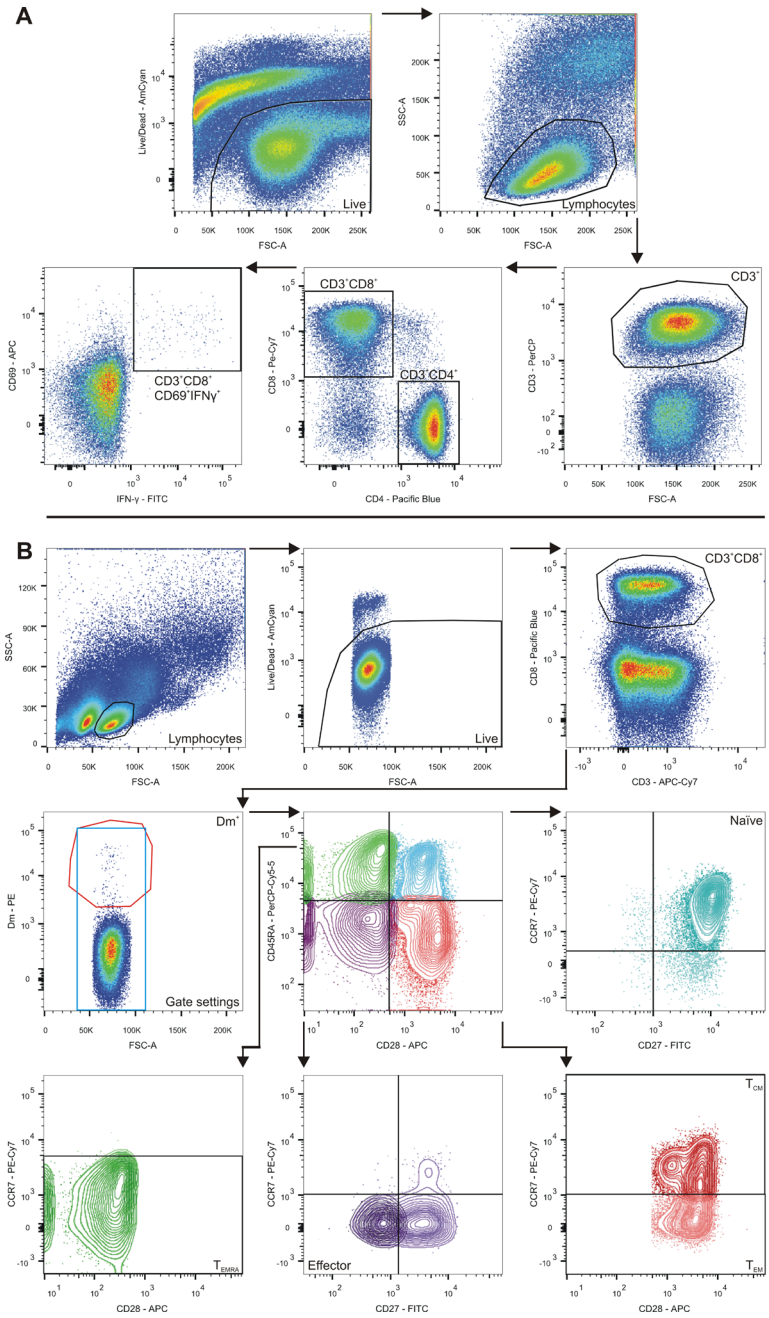

Figure 1 Defining influenza A virus-specific CD8⁺ T cells using flow cytometry.
A, The frequency of IAV-specific CD8⁺ T cells was determined after stimulating peripheral blood mononuclear cells with IAV H3N2. Fluorochrome-labeled antibodies were used to identify the CD8⁺ T-cell population expressing CD3, interferon γ, and CD69. Background values of nonstimulated control cells were subtracted. B, In addition, the frequency of IAV-specific CD8⁺ T cells was determined using Dextramer (Dm) staining (red gate). Various T-cells subsets were further assessed on the basis of expression of CD45RA, CD28, CCR7, and CD27. Owing to the low frequency of CD8⁺Dm⁺ T cells, the gating strategy was based on the whole CD3⁺CD8⁺ T-cell population (blue gate).

buffered saline containing 5% fetal bovine serum (Sigma-Aldrich) and incubated for 10 minutes at room temperature with the Dm mixture. $CD8^+Dm^+$ cells were further functionally phenotyped as naïve cells ($CD45RA^+CD28^+CCR7^+CD27^+$), effector cells ($CD45RA^-CD28^-CCR7^-CD27^-$), effector memory T cells (T_{EM}) ($CD45RA^-CD28^+CCR7^-$), effector memory RA T cells (T_{EMRA}) ($CD45RA^+CD28^-CCR7^-$) and central memory T cells (T_{CM}) ($CD45RA^-$ $CD28^+CCR7^+$) using fluorochrome-labeled antibodies directed to the respective CD antigens (BD Biosciences, Breda, The Netherlands and eBiosciences, Vienna, Austria) (Figure 1B). Remaining cells were defined as "other" and consist of cell subsets which have not been defined previously e.g. $CD45RA^-CD28^-CCR7^-CD27^+$ or $CD45RA^-CD28^-CCR7^+CD27^+$.

Results

Based on the Dm staining, donors 4564, 7482, 5878, 6358 and 5891 display an increase in number of IAV-specific $CD8^+$ T cells at one time point (Figure 2A, B, C, E and I). Expansion of effector T-cell, T_{EM} and T_{EMRA} populations mainly accounted for this increase, which is typically observed after a recent infection [332]. For three of these donors (4564, 5878 and 6358) the increase in IAV-specific $CD8^+$ T-cell numbers coincided with an antibody response directed against contemporary IAV strains (H3N2 and/or H1N1), suggesting that IAV-infection was responsible for the increase in IAV-specific CTLs (Figure 2A, C and E). However, donor 5891 did not seroconvert, despite an increase in IAV-specific CTL numbers (Figure 2I). Of note, since the PBMCs and corresponding plasma sample were obtained early in 2010, a possible seroconversion later that year could not be excluded (Supplementary Table 1). Unfortunately, for donor 7482 no plasma sample was available for the year 2008, which precluded correlating T-cell and antibody responses (Figure 2B).

A subsequent decrease in the frequency of IAV-specific $CD8^+$ T cells was found in donors 4564, 7482, 5878 and 6358 (Figure 2A, B, C and E) which was accompanied with a contraction of the effector T cell, T_{EM} and T_{EMRA} subsets. In years following the contraction phase, small numbers of T_{EM}, T_{EMRA} and T_{CM} persisted in these study subjects.

As shown by Dm-staining and ICS, the frequency of IAV-specific $CD8^+$ T cells remained relatively stable over the years in the other 4 donors (8801, 6888, 8904 and 6877), although VN antibody testing indicated a possible IAV infection for donor 6888 in 1998/99, this did not correspond with a conclusive increase in IAV-specific $CD8^+$ T cells (Figure 2D, F, G and H)

In most cases (4564, 7482, 8801, 6358, 6888, 8904 and 6877) the results obtained with Dm-staining and ICS correlated well. For donors 5878 and 5891 the correlation was less obvious. Since we used a cocktail of selected Dms to stain influenza virus-specific $CD8^+$ T cells, it is likely that $CD8^+$ T cells with specificity for other (unknown) epitopes were not detected, so the use of a Dm cocktail may have underestimated the number of IAV-specific $CD8^+$ T cells, especially in case of donors

8801 and 6877.

Discussion

Although PBMC and/or plasma samples were not available for each donor and every year, these data indicate for the first time that the human IAV-specific $CD8^+$ T-cell immunity persists for a prolonged period of time. Although indication of recent infections were not found for all donors tested (7482, 8801, 8904, 6877 and 5891), it is likely that all subjects experienced multiple infections with A/H1N1 and A/H3N2 IAV since childhood [287]. The various $CD8^+$ T-cell subsets are relatively stable over the years. However, IAV infection may induce a transient increase in the frequency of IAV-specific $CD8^+$ T cells, which can mainly be attributed to an increase of effector T cell, T_{EM} and T_{EMRA} subsets. Of interest, the proportion of these subsets decrease in the contraction phase. The proportion of IAV-specific $CD8^+$ T cells as detected by Dm-staining and ICS is small, but comparable to that of memory T cells against other viruses causing acute infections [606, 607]. Only during chronic virus infections larger virus-specific T-cell populations may be observed [608]. Of note, none of our study subjects experienced an acute IAV infection at the sampling time points, as illness in the two weeks prior to blood donation is an exclusion criteria. The contraction of IAV-specific $CD8^+$ T-cell response occurs rapidly within one to two weeks post clinical onset as was demonstrated in patients acutely infected with the 2009 pandemic H1N1 virus [603] and similar contraction of $CD8^+$ T cells was demonstrated after vaccination with live attenuated yellow fever (YFV-17D) and smallpox (Dryvax) vaccines [607]. The real number of persisting T_{CM} may be higher than shown in figure 2, since these cells preferentially reside in the lymphnodes rather than in peripheral blood [332].

Collectively, we demonstrated that adult subjects possess IAV-specific $CD8^+$ T cells and that the presence of this cell-mediated immunity in the blood is long-lived. Since the majority of these T cells are highly cross-reactive, they will respond to infection with antigenically related and unrelated IAVs. The presence of these cells correlated with protection against severe disease caused by IAV, as was shown recently [344]. Thus, repeated boosting of IAV-specific cross-reactive $CD8^+$ T-cell responses, for example by the use of live attenuated vaccines or alternative T-cell antigen delivery systems, may be a venue to induce broadly protective immunity against future pandemic influenza viruses.

Acknowledgements

We thank R.D. de Vries for excellent technical advice and assistance.

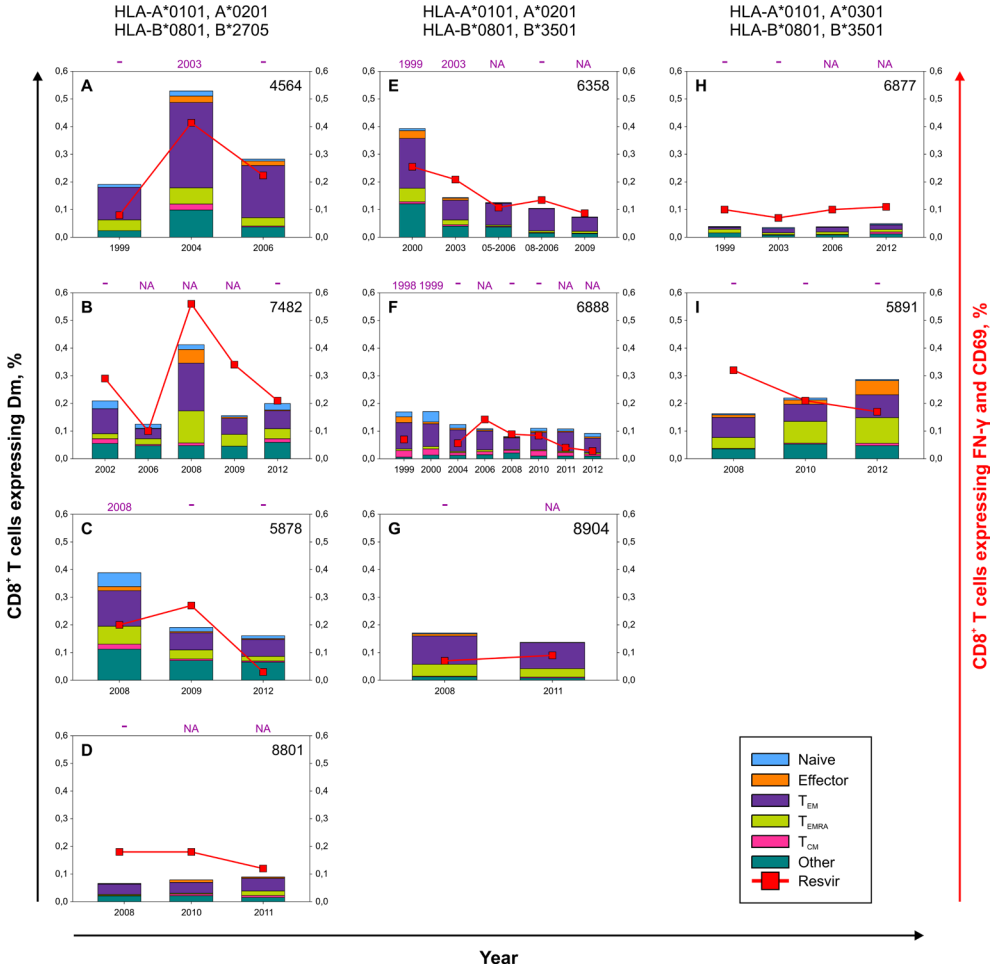

Figure 2 Phenotyping influenza A virus (IAV)-specific CD8+ T cells.
The frequency of IAV-specific CD8⁺ T cells was determined in peripheral blood mononuclear cells (PBMCs) obtained from healthy blood donors in the indicated years. Bars indicate the frequency of Dextramer (Dm)-expressing CD8⁺ T cells (left Y-axis). Based on the expression of CD45RA, CD28, CCR7, and CD27, the IAV-specific CD8⁺Dm⁺ T cells were further subdivided into various T-cell subsets to determine the proportion of naïve T cells, effector T cells, effector memory T cells (T_{EM}), effector memory RA T cells (T_{EMRA}), and central memory T cells (T_{CM}). Cells not belonging to any of these subsets were defined as "other". The red line indicate the frequency of IAV-specific CD8⁺ T-cell population expressing interferon γ (IFN-γ) and CD69, as determined by intracellular IFN-γ staining, after stimulation with infectious IAV (right Y-axis). Above each graph, the year of isolation is indicated for the IAV that circulated during influenza season preceding the time point of PBMC collection and against which antibody responses were detected by a virus neutralization assay. NA, plasma sample was not available for that time point. -, no seroconversion against an IAV strain circulating in the preceding influenza season was detected.

Supporting Material for

Human influenza A virus-specific CD8$^+$ T-cell response is long-lived

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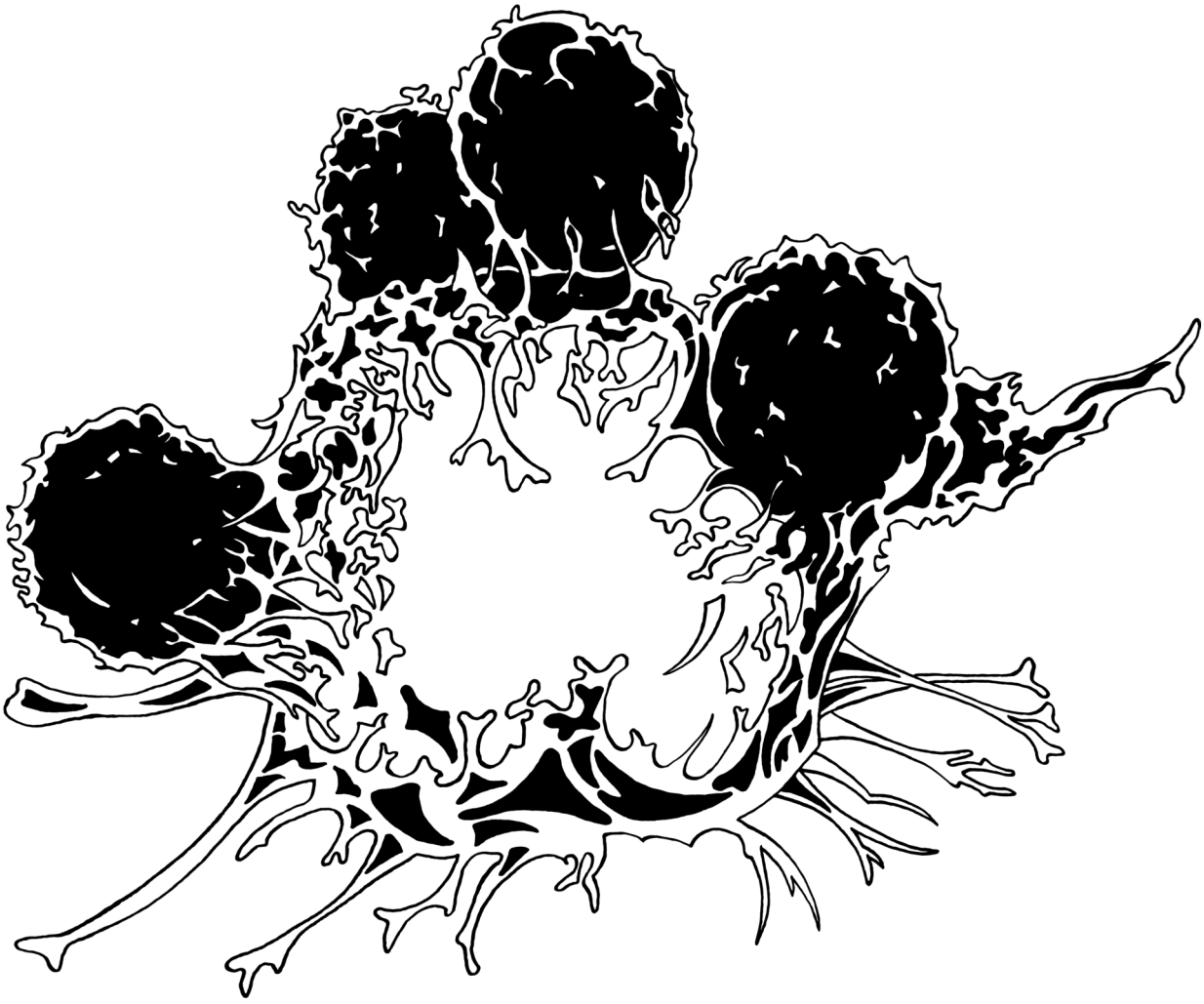
Supplementary table 1 HLA-typed blood donors

			Time Point							
Group	**Donor**	**HLA-Type**	**T=1**	**T=2**	**T=3**	**T=4**	**T=5**	**T=6**	**T=7**	**T=8**
1	4564	HLA-A*0101, A*0201, B*0801, B*2705	17-09-1999	17-03-2004	16-05-2006					
	7482	HLA-A*0101, A*0201, B*0801, B*2705	07-02-2002	20-03-2006*	18-03-2008*	27-10-2009*	21-03-2012			
	5878	HLA-A*0101, A*0201, B*0801, B*2705	18-11-2008	16-10-2009	11-12-2012					
	8801	HLA-A*0101, A*0201, B*0801, B*2705	27-11-2008	22-01-2010*	11-02-2011					
2	6358	HLA-A*0101, A*0201, B*0801, B*3501	20-04-2000	25-02-2003	18-05-2006	10-08-2006	03-12-2009*			
	6888	HLA-A*0101, A*0201, B*0801, B*3501	07-04-1999	27-04-2000	22-10-2004	12-05-2006*	04-09-2008	25-11-2010	18-08-2011*	22-05-2012*
	8904	HLA-A*0101, A*0201, B*0801, B*3501	27-11-2008	14-07-2011*						
3	6877	HLA-A*0101, A*0301, B*0801, B*3501	07-04-1999	25-02-2003	15-03-2006	19-09-2012*				
	5891	HLA-A*0101, A*0301, B*0801, B*3501	18-11-2008	09-03-2010	21-03-2012					

* No plasma available for VN assay



CHAPTER 5:

Differential recognition of influenza A viruses by $M1_{58-66}$ epitope-specific $CD8^+$ T cells is determined by extra-epitopic amino acid residues

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Abstract

Natural influenza A virus infections elicit both virus-specific antibody and $CD4^+$ and $CD8^+$ T cell responses. Influenza A virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) contribute to clearance of influenza virus infections. Viral CTL epitopes can display variation, allowing influenza A viruses to evade from recognition by epitope-specific CTLs. Due to functional constraints, some epitopes, like the immunodominant HLA-A*0201 restricted matrix protein 1 $(M1)_{58\text{-}66}$ epitope, are highly conserved between influenza A viruses regardless of their subtype or host species of origin. We hypothesized that human influenza A viruses evade recognition of this epitope by impairing antigen processing and presentation by extra-epitopic amino acid substitutions. Activation of specific T cells was used as an indication for antigen presentation. Here, we show that the $M1_{58\text{-}66}$ epitope in the M1 protein derived from human influenza A virus was poorly recognized compared to the M1 protein derived from avian influenza A virus. Furthermore, we demonstrated that naturally occurring variation at extra-epitopic amino acid residues affect $CD8^+$ T cell recognition of the $M1_{58\text{-}66}$ epitope. These data indicate that human influenza A viruses can impair recognition by $M1_{58\text{-}66}$-specific CTLs, while retaining the conserved amino acid sequence of the epitope, which may represent a yet unknown immune evasion strategy for influenza A viruses. This difference in recognition may have implications for the viral replication kinetics in HLA-A*0201 individuals and spread of influenza A viruses in the human population. The findings may aid the rational design of universal influenza vaccines that aim at the induction of cross-reactive virus-specific CTL responses.

Importance

Influenza viruses are an important cause of acute respiratory tract infections. Natural influenza A virus infections elicit both humoral and cellular immunity. $CD8^+$ cytotoxic T lymphocytes (CTLs) are directed predominantly against conserved internal proteins and confer cross-protection, even against influenza A viruses of various subtypes. In some CTL epitopes mutations occur that allow influenza A viruses to evade from recognition by CTLs. However, the immunodominant HLA-A*0201 restricted $M1_{58\text{-}66}$ epitope does not tolerate mutations without loss of viral fitness. Here, we describe naturally occurring variations in amino acid residues outside the $M1_{58\text{-}66}$ epitope that influence the recognition of the epitope. These results provide novel insights in the epidemiology of influenza A viruses and their pathogenicity and may aid rational design of vaccines that aim at the induction of CTL responses.

Introduction

Influenza viruses are among the leading causes of acute respiratory tract infections worldwide [65]. Classification of influenza A viruses (IAVs) is based on their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). At present, 18 HA (H1-H18) and 11 NA (N1-N11) subtypes have been identified [2, 4]. IAVs of the H3N2 and H1N1 subtype together with influenza B viruses cause yearly epidemics in the human population [65]. Other IAV subtypes circulate in animal reservoirs like aquatic birds and pigs [53], but can occasionally cross the species barrier into the human population [609]. Genetic reassortment between animal and human IAVs have resulted in the emergence of pandemic strains in the last century [81-84].

Natural influenza virus infections elicit both humoral and cellular immune responses. Virus neutralizing antibodies are mainly directed against the highly variable globular head of the HA protein and prevent re-infection with the same virus [610]. However, most antibodies have limited cross-reactivity against influenza viruses of another subtype [68, 69] and may afford little protection against the development of severe disease caused by infection with antigenically distinct viruses, including those of novel subtypes.

Influenza virus-specific $CD8^+$ T cells (cytotoxic T-lymphocytes (CTLs)) on the other hand are directed predominantly against more conserved internal proteins [528, 611] and recognize their epitopes as major histocompatibility (MHC) class I/peptide complexes [411]. The recognition of conserved proteins results in a high degree of cross-reactivity with antigenically distinct IAVs [316, 528, 536, 611]. Although CTLs do not afford sterilizing immunity, they contribute substantially to viral clearance and reduce disease severity of infections with influenza viruses including those with antigenically distinct HA or NA [344, 612, 613]. However, the high mutation rate of influenza viruses and the selective pressure exerted by virus-specific CTLs drive the accumulation of amino acid substitutions that are associated with evasion from recognition by CTLs specific for some epitopes. Indeed, significantly more non-synonymous mutations are observed in CTL epitopes than in the rest of the viral nucleoprotein (NP) [410, 614]. Amino acid substitutions in T cell receptor (TCR) contact residues have been identified that result in loss of recognition by epitope-specific CTLs [422, 528], as has been described for the human leukocyte antigen (HLA)-B*3501 restricted $NP_{418-426}$ epitope [427]. In addition, mutations at anchor residues of CTL epitopes have been identified [422, 528], which resulted in complete loss of the CTL epitope as has been described for the HLA-B*2705 restricted $NP_{383-391}$ epitope [421, 425]. Both types of CTL escape mutations were observed during natural evolution of seasonal IAVs (H3N2) [422, 425]. Similar CTL evasion strategies have been described for viruses that cause chronic infections, like Human Immunodeficiency Virus (HIV) [615], Hepatitis C Virus (HCV) [436], Epstein-Barr Virus (EBV) [616] and Lymphocytic Choriomenigitis Virus (LCMV) [439]. In contrast, some IAV CTL epitopes are highly conserved even between different

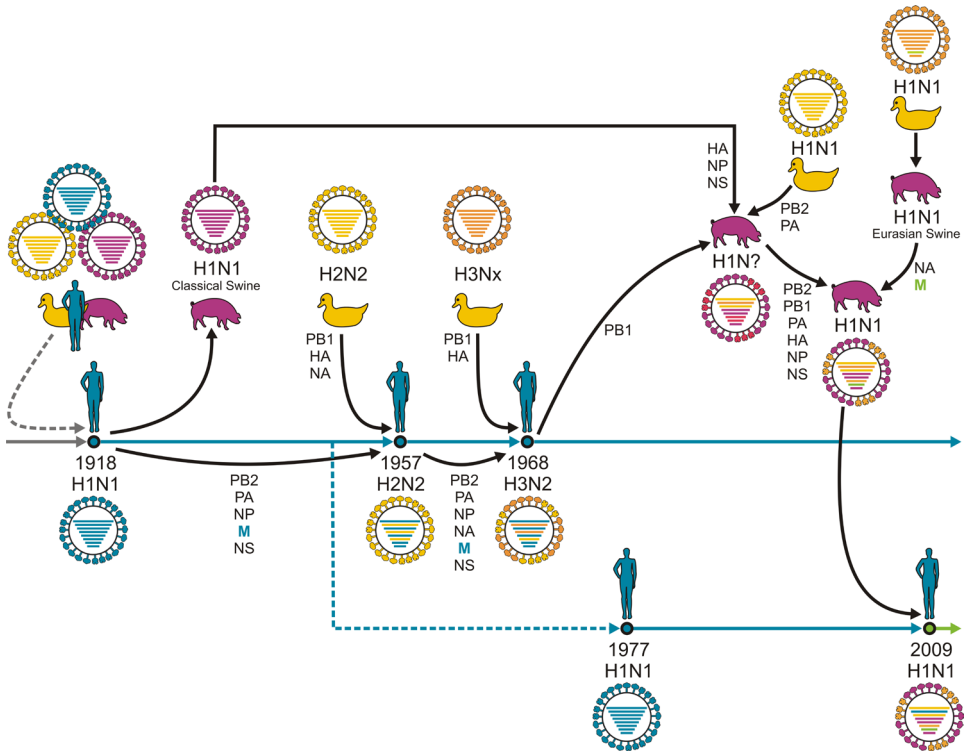

Figure 1 Reassortment events of pandemic influenza A viruses
The 1918 A/H1N1 virus possibly originated from multiple reassortment events between avian, swine and human viruses. This A/H1N1 virus continued to circulate, causing seasonal epidemics, until 1957 when a novel A/H2N2 virus emerged after a reassortment event with an avian A/H2N2 virus. This virus circulated until 1968, when it reassorted with an avian A/H3Nx virus, and caused seasonal epidemics ever since. A/H1N1 was reintroduced in the human population in 1977 and co-circulated with A/H3N2 viruses until 2009 when it was replaced by H1N1pdm2009, which originated after multiple reassortment events between avian, swine and human viruses. Although it is unknown whether the M gene segment originated from viruses that were newly introduced into humans or had circulated in humans prior to 1918, viruses with this gene segment continued to circulate in the human population in A/H2N2 and A/H3N2 viruses until today (blue arrow). Also, the A/H1N1 virus that was reintroduced into the human population in 1977 contained the M gene segment of 1918 origin, but this virus was replaced by a virus with a swine derived M gene segment during the A/H1N1 pandemic outbreak of 2009 (green arrow).

subtypes of IAV, like the HLA-A*0201 / HLA-C*0801 restricted $M1_{58-66}$ (GILGFVFTL) epitope [446, 447]. The matrix protein 1 (M1) of seasonal A/H3N2 viruses originates from the 1918 pandemic A/H1N1 virus (Fig. 1) [81-83, 453, 454]. Most likely, the selective pressure against the $M1_{58-66}$-epitope is high, considering the immunodominant nature of the epitope [448] and the high prevalence of the HLA-A*0201 allele in the Caucasian population (>40%) [617]. However, mutations at TCR contact or anchor residues were not tolerated in this epitope without loss of viral fitness [410, 450], which coincides with the presence of a highly conserved nuclear export signal overlapping the $M1_{58-66}$ epitope [451].
We hypothesized that IAVs may have adopted other escape mechanisms for highly conserved CTL epitopes, like the $M1_{58-66}$ epitope, based on the observation

that avian IAVs of the H5N1 and H7N9 subtype are better recognized by polyclonal IAV-specific CTLs than human seasonal IAVs [316, 536]. It is well known that amino acid substitutions flanking an epitope can affect antigen presentation by changing the cleavage motifs used by the proteasome, alter trimming of the N- and C-terminal sequence by cytosolic or endoplasmic reticulum (ER) resident proteases or impair the translocation via TAP (transporter associated with antigen presentation) [411, 413, 414]. So far, an effect of extra-epitopic mutations on T cell recognition has only been demonstrated for CTLs directed to viruses that cause chronic infections, including for HIV [615], HCV [418] and EBV [419, 420].
Here we investigate if differences in extra-epitopic amino acid residues observed between avian and human IAVs could be responsible for differential recognition of the $M1_{58-66}$ epitope. It was demonstrated that naturally occurring amino acid differences at positions in the region flanking the otherwise fully conserved $M1_{58-66}$ epitope affect recognition by $M1_{58-66}$-specific $CD8^+$ T cells. The reduced recognition of human IAVs by $M1_{58-66}$-specific $CD8^+$ T cells by extra-epitopic amino acid substitutions indicates the existence of an immune evasion strategy additional to variation in CTL epitopes and may help the virus to perpetuate in the human population in the presence of pre-existing virus-specific $CD8^+$ T cell immunity. Furthermore, these results are of interest for the development of vaccines that aim at the induction of virus-specific CTL responses.

Materials and Methods

Cells

A HLA-A*0101/A*0201/B*0801/B*2705 B lymphoblastoid cell line (BLCL) was prepared as described previously [513]. BLCLs were cultured in RPMI 1640 medium (Lonza, Basal, Switzerland) supplemented with 100µg/ml penicillin, 100U/ml streptomycin, 2mM L-Glutamine (P/S/G) (Lonza) and 10% fetal bovine serum (FBS) (Sigma-Aldrich, Zwijndrecht, The Netherlands) (R10F medium).
The previously described A549-HLA-A*0201^+ human lung carcinoma cell line [618] was cultured in HAMs-F12 medium (Gibco Life Technologies, Bleiswijk, The Netherlands) containing P/S/G, 10% fetal calf serum (FCS) (Hycone) (Sigma) (H10F) and in the presence of 1µg/ml puromycin (Invivogen, Toulouse, France). HLA-A*02 expression was confirmed by staining with anti-HLA-A*02-FITC (BD Biosciences, Breda, The Netherlands) and using a FACSCantoII flowcytometer and FACS Diva software (Becton Dickinson B.V., Breda, The Netherlands), prior to each experiment.

Peptides

Synthetic immunograde peptides (>85% purity) of the HLA-A*0201 restricted $M1_{58-66}$ (GILGFVFTL) and the HLA-B*2705 $NP_{383-391}$ (SRYWAIRTR) epitopes were purchased (Eurogentec, Seraing, Belgium). Peptides were dissolved in dimethyl sulfoxide (5 mg/ml), diluted to 100 µM in RPMI 1640 medium and stored at -20°C until further use.

Plasmids

The open reading frame (ORF) of the M1 protein of influenza viruses A/Netherlands/018/1994 (H3N2) or A/Vietnam/1194/2004 (H5N1) without their stop codons were cloned in frame with the ORF of eGFP into the pEGFP-N1 plasmid (Becton Dickinson) as described previously [619]. Next, the ORF of the $NP_{383-391}$ (SRYWAIRTR) epitope including 50 N- and C- terminal amino acids (all derived from A/PuertoRico/8/1934) was cloned in frame in between the ORFs of the M1 and eGFP protein (Fig. 2). Briefly, the NP insert (nucleotide (nt) position 997 to 1323) was created by PCR amplification of A/PuertoRico/8/1934 derived NP cDNA using a forward and reverse primer that encompassed 20 nts of the vector and 20 nts of the desired NP insert. These primers were used in the following PCR reaction: 10 pmol of each primer, 5 µl of pfu ultra II buffer, 1 µl of *pfu ultra II* enzyme (Agilent Technologies, Amstelveen, The Netherlands), dNTP (10 mM each) (Roche, Woerden, The Netherlands), 100 ng A/PuertoRico/8/1934 NP gene segment cDNA in a final volume of 50 µl, which was subsequently incubated at 95°C for 3 minutes (min), followed by 40 cycles of 95°C for 1 min, 1 min at 45°C and 72°C for 2 min. PCR products were loaded on a 1% agarose gel and DNA was isolated using the min elute gel extraction kit from Qiagen according to the manufacturer's instructions (Qiagen, Venlo, The Netherlands). This purified PCR product now served as a 'megaprimer'. The second PCR was performed as described above, only this time 100, 300, and 500 ng of the 'megaprimer' was combined with 50 and 100 ng of vector DNA, and 3 µl quik solution (Agilent). The PCR product was digested for 1 hr at 37°C with 20U of *DpnI* (New England Biolabs, Ipswich, USA). The DpnI digested PCR product was transformed using Z-competent XL-10 gold cells (Zymo research, Irvine, USA). Plasmid DNA was purified using a Genopure Plasmid Maxi Kit (Roche). Reciprocal exchange of the extra-epitopic amino acids in the M1 protein at positions 15, 27, 101, 115 and 121 were introduced using the Quikchange multi site-directed mutagenesis kit according to the manufacturer's instructions (Agilent Technologies) (Fig. 2). These plasmids were used in the FATT-CTL assay.

A bidirectional reverse genetic system based on influenza virus A/Netherlands/178/1995 (H3N2; M), A/Vietnam/1194/2005 (H5N1; M) and A/PuertoRico/8/1934 (H1N1; PB2, PB1, PA, HA, NP, NA, NS) were used for the generation of recombinant influenza viruses as described previously [410, 620, 621]. Sequences of all recombinant plasmids were confirmed by sequence analysis using a Big Dye Terminator v3.1 cycle sequencing kit and a 3130xl genetic analyzer (Applied Biosystems, Bleiswijk, The Netherlands) prior to use.

Viruses

293T cells were transfected with the recombinant bidirectional plasmids (M derived from A/Netherlands/178/1995 or A/Vietnam/1194/2005, other gene segments derived from A/Puerto Rico/8/1934) as described previously [620]. Culture

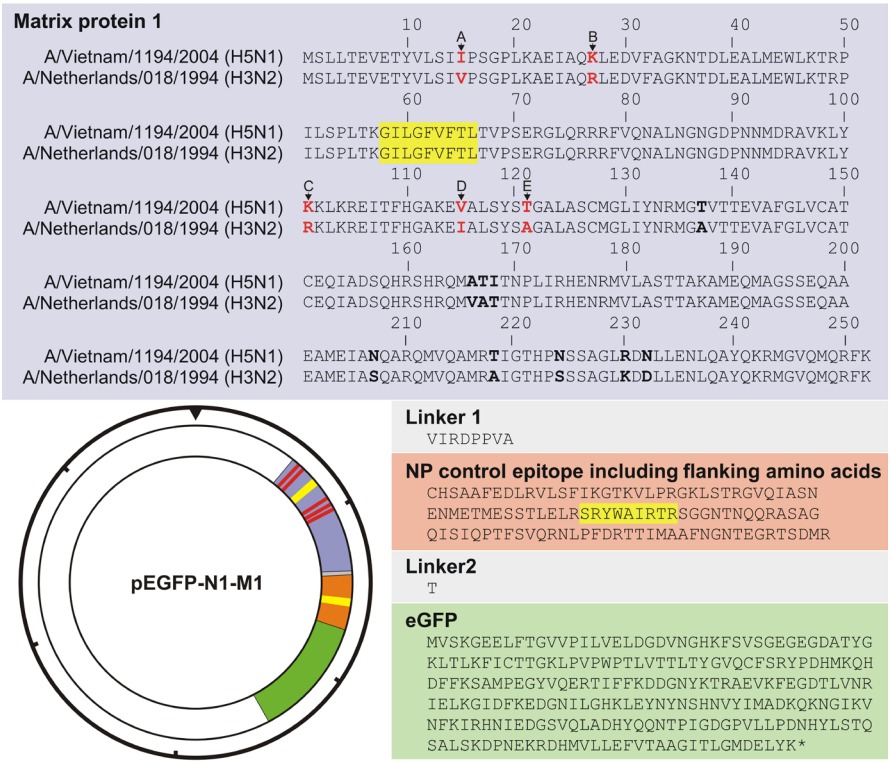

Figure 2 Amino acid sequences of viral M1-NP-eGFP fusion proteins and expression plasmid map
The amino acid sequence of the chimeric M1-NP-eGFP fusion construct is shown with the avian IAV A/Vietnam/1194/2004 (H5N1) and human IAV A/Netherlands/018/1994 (H3N2) M1 encoding sequences in blue, NP in orange and eGFP in green. Linker sequences are shown in gray. The location of the $M1_{58-66}$ (GILGFVFTL) and $NP_{383-391}$ (SRYWAIRTR) epitopes are highlighted in yellow. Amino acid differences studied in this paper are indicated in bold red, additional amino acid differences are indicated in bold. The insert was cloned into the pEGFP-N1 vector as indicated. The hash marks around the perimeter of the plasmid map indicate 1000 nucleotide increments.

supernatants were harvest after 48 hrs and used for a subsequent inoculation of Madin-Darby canine kidney (MDCK) cells [421]. After 3 days, culture supernatants were harvested and passed twice in MDCK cells. Culture supernatants were clarified by low-speed centrifugation and subsequently purified by ultracentrifugation through a sucrose gradient. Sequence analysis was used to confirm the sequence of the M gene segments as described above and their infectious-virus titers were determined as described previously [545].
Note that the M gene segment of the recombinant virus was derived from an alternative A/H3N2 virus (A/Netherlands/178/1995) which differed from the A/Netherlands/018/1994 virus at amino acid positions 227 and 239 (A227T and A239T). However, we argue that due to the large C-terminal distance (>160 amino acids) from the $M1_{58-66}$ epitope these amino acid differences are unlikely to interfere with the processing of this epitope.

T cell clones

CD8+ T cell clones directed against the HLA-A*0201-restricted $M1_{58-66}$ (GILGFVFTL) epitope and the HLA-B*2705-restricted $NP_{383-391}$ (SRYWAIRTR) epitope were generated as described previously [425, 622].

FATT-CTL assay

The fluorescent antigen-transfected target cell (FATT)-CTL assay was used for the detection of lytic activity of the $M1_{58-66}$- and $NP_{383-391}$-specific CD8+ T cell clones as described previously [619]. Briefly, the cell line nucleofector kit V (Lonza), program T16, was used to transfect $5x10^6$ BLCLs with 8µg plasmid DNA and incubated in R10F for 4 hrs at 37°C. The number of viable eGFP-positive cells was determined after TOPRO®-3 iodide (Invitrogen, Breda, The Netherlands) staining using the FACSCantoII flowcytometer and FACS Diva software. Quadruples of 1,500 viable eGFP-positive target cells were cocultured for another 3.5 hrs with 20,000, 40,000 or 80,000 $M1_{58-66}$- or $NP_{383-391}$-specific CD8+ T cells and the number of viable eGFP-positive cells was determined as described above. The gating strategy performed with FlowJo software (FlowJo, Ashland, USA) is shown in figure 3A. The percentage of epitope-specific lysis was then calculated using the following formula: 100 x [(number of viable eGFP-positive cells in the sample without effector – number of viable eGFP-positive cells in the sample with effector)/number of viable eGFP-positive cells in the sample without effector].

Kinetics of CD8+ T cell activation in the FATT-CTL assay

BLCLs were transfected and counted as described above. Quadruples of 3,000 viable eGFP-positive target cells were cocultured for another 7 hrs with 10,000 or 20,000 $M1_{58-66}$- or $NP_{383-391}$-specific CD8+ T cells in the presence of golgistop [4µl/6ml] (BD Biosciences) and 0.5µl/100µl CD107a-V450 (BD Biosciences) and subsequently stained in phosphate buffered saline (PBS) supplemented with 2% FBS and golgistop (P2FG) with CD3-PerCP-Cyanine5.5, CD8-APC (eBiosciences, Vienna, Austria), CD137-PECyanine7 (BioLegend, London, United Kingdom), CD69-APC-H7 (BD Biosciences) and LIVE/DEAD aqua Fixable Dead cell stain (L/D) (Invitrogen). Next, cells were fixed using Cytofix (BD Biosciences) and stored in PBS supplemented with 0.5% BSA and 2mM Ethylenediaminetetraacetic acid (EDTA) (Sigma) at 4°C until they were analyzed using a FACSCantoII flowcytometer and FACS Diva software. Gating strategy using FlowJo software is shown in figure 4A. BLCLs pulsed with or without 100µM GILGFVFTL or SRYWAIRTR peptide were used as a positive control (data not shown).

IFN-γ ELISpot assay

The interferon gamma (IFN-γ) response of $M1_{58-66}$- or $NP_{383-391}$-specific CD8+ T cells was determined by enzyme-linked immunosorbent spot (ELISpot) assay, which was

performed according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden). In brief, 3,000 transfected viable eGFP-positive BLCLs were incubated with 10,000 $M1_{58-66}$- or $NP_{383-391}$-specific $CD8^+$ T cells for 7 hrs, in quadruplicate. The average number of spots was determined using an ELISpot reader and image analysis software (Aelvis, Sanquin Reagents, Amsterdam, The Netherlands).

Virus infection and kinetics $CD8^+$ T cell activation

The kinetics of $M1_{56-66}$-specific $CD8^+$ T cells activation after stimulation with A549-HLA-A*0201$^+$ cells infected with the avian or human recombinant viruses was studied by assessing expression of activation markers CD137, CD69 and CD107a. Peptide pulsed A549-HLA-A*0201$^+$ cells were used as a positive control.
A549-HLA-A*0201$^+$ cells were incubated with or without 100µM GILGFVFTL in H10F for 1hr at 37°C in an ultra-low attachment plate (Corning, New York, USA). Meanwhile, virus-infected target cells were prepared by inoculating A549-HLA-A*0201$^+$ cells at a multiplicity of infection (MOI) of 3 with the avian or human recombinant virus in ultra-low attachment plates. After 1 hr, cells were washed with H10F and cocultured with the $M1_{56-66}$-specific $CD8^+$ T cell clone at an E:T ratio of 0.2 in the presence of golgistop [4µl/6ml] and 0.5µl/100µl CD107a-V450, in triplicate for each timepoint. Cells were stained each hr from 3 till 14 hr p.i. and 24 hr p.i. with CD8-FITC (Dako, Glostrup, Denmark), CD137-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), CD3-PerCP, CD69-APC (BD Biosciences) and L/D and subsequently fixed and stored as described above. Virus-infected A549-HLA-A*0201$^+$ cells in the absence of the $M1_{56-66}$-specific $CD8^+$ T cell clone were simultaneously stained with L/D and subsequently fixed and permeabilized with Cytofix and Cytoperm (BD Biosciences) after which the cells were stained for 30 min at 4°C with anti-influenza A-FITC (reagent A) (Oxoid, Landsmeer, The Netherlands). Cells were analyzed using a FACSCantoII flowcytometer and FACS Diva software. Gating strategy using FlowJo software is shown in figure 6A and B.

Sequence Data

To assess the frequency of amino acid variations in the M1 protein at positions 15, 27, 101, 115 and 121 in avian (all subtypes available 2001-2015), swine (A/H3N2 1977-2015 and A/H1N1 1930-2015) and human (A/H1N1 1918-1957, A/H2N2 1957-1968, A/H3N2 1968-2015, A/H1N1 1977-2008 and A/H1N1 2009-2015) viruses, all full length M1 amino acid sequences available in the influenza virus resource database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genomes/FLU) at April 11th 2015 were downloaded. Due to the large number of avian viruses available we collapsed all identical sequences prior to analysis. After excluding sequences with large deletions using BioEdit we analyzed the dataset in Ugene 1.16.1 (http://ugene.unipro.ru; Unipro, Novosibirsk, Russia) to assess the frequency

of the avian or human amino acids at positions 15, 27, 101, 115 and 121. Viruses were analyzed in Excel to determine whether observed frequencies were the result of cluster formation and whether certain mutations became fixed in time.

Statistical analysis

Data from the FATT-CTL, ELISpot and activation assays were analyzed using the independent samples T test to calculate the respective *p* value between pairs of groups. These *p* values were then analyzed using the Benjamini-Hochberg method (a false discovery rate (FDR) of 0.01 was used for all assays) to correct for multiple hypothesis testing [623]. Each experiment, with the exception of the IFN-γ ELISpot assay, was performed at least twice.

Results

Differences in lytic activity of $M1_{58-66}$-specific $CD8^+$ T cells against M1 proteins derived from avian and human influenza A viruses

Viruses were selected based on the previous observation that avian IAV A/Vietnam/1194/2004 (H5N1) was better recognized by IAV-specific CTLs than human IAV A/Netherlands/018/1994 (H3N2) [316] which may be attributable to a yet unidentified, additional CTL escape mechanism utilized by human A/H3N2 viruses.
The M1 genes of both viruses were cloned in frame with the enhanced green fluorescent protein (eGFP) gene into an expression plasmid as described previously [619]. In addition, a region of NP, encoding the HLA-B*2705 $NP_{383-391}$ (SRYWAIRTR) epitope and 50 N- and C-terminal flanking amino acids, was cloned in frame between the M1 and eGFP genes (Fig. 2). The $NP_{383-391}$ epitope was included as a control since CTLs specific for this epitope have similar functional avidity as CTLs directed to the $M1_{58-66}$ epitope [448]. These plasmids were used in the FATT-CTL assay to monitor the lytic activity by $M1_{58-66}$-specific $CD8^+$ T cells (Fig. 3A), as described previously [619]. Lytic activity of the $NP_{383-391}$-specific $CD8^+$ T cells was used as a control to exclude differences in transfection efficiencies and/or protein expression levels. $M1_{58-66}$-specific $CD8^+$ T cells lysed significantly more target cells expressing the M1 protein derived from avian virus A/Vietnam/1194/2004 (H5N1) than those expressing the M1 protein of human virus A/Netherlands/018/1994 (H3N2) (83% and 45% respectively at the highest effector:target (E:T) ratio) (Fig. 3B). The lytic activity of $NP_{383-391}$-specific $CD8^+$ T cells to both M1-NP-eGFP fusion proteins was similar (70% at the highest E:T ratio) (Fig. 3C). These results demonstrate that the $M1_{58-66}$ epitope in context of a M1 protein derived from a human IAV is less well recognized than its counterpart in the context of a M1 protein derived from an avian IAV. Next, we wished to assess whether differences in amino acids flanking the $M1_{58-66}$ epitope had contributed to the observed difference in lytic activity of epitope-specific $CD8^+$ T cells. Although no amino acid differences were found in close proximity to the $M1_{58-66}$ epitope, we identified five extra-epitopic (avian-to-human) amino acid

substitutions within a 60 amino acid distance of the $M1_{58-66}$ epitope; namely at positions I15V (substitution A), K27R (substitution B), K101R (substitution C), V115I (substitution D) and T121A (substitution E) (Fig. 2). Reciprocal exchange of these extra-epitopic amino acid residues in the M1 protein allowed assessment of the effect of these substitutions on recognition by $M1_{58-66}$-specific $CD8^+$ T cells. Exchanging the extra-epitopic amino acid residues partially reversed the recognition pattern of the $M1_{55-66}$-specific $CD8^+$ T cells. Introducing the five M1 amino acid residues of the human virus into the M1 protein of the avian virus (A→H ABCDE) significantly reduced the lytic activity of the $M1_{58-66}$-specific $CD8^+$ T cells from 83% to 70% at the highest E:T ratio. Introducing the five M1 amino acid residues of the avian virus into the M1 protein of the human virus (H→A ABCDE) significantly improved the lytic activity of $M1_{58-66}$-specific $CD8^+$ T cells from 45% to 56% (Fig. 3B). The H→A ABCDE exchange did not affect the recognition by the $NP_{383-391}$-specific $CD8^+$ T cells, while the A→H ABCDE exchange affected recognition slightly (Fig. 3C). The five amino acid differences are unlikely to have altered the CTL response by interfering with the splice site, as this would have resulted in a shift of the NP open reading frame (ORF) [624], resulting in comparable recognition patterns by both $CD8^+$ T cell clones, which was not observed.

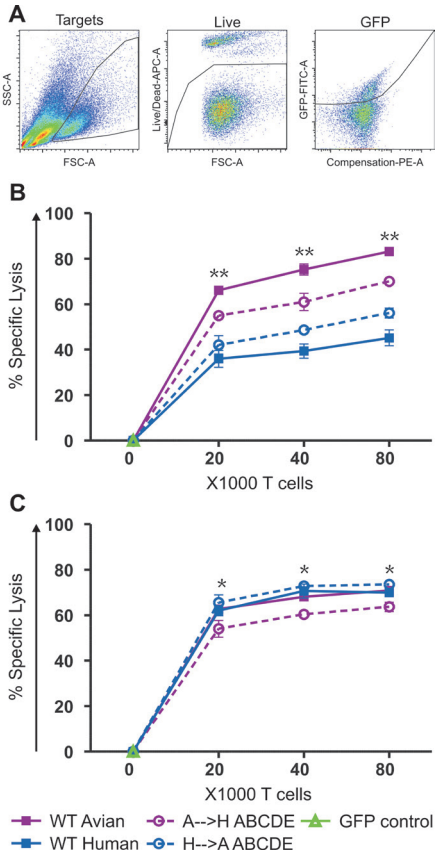

Figure 3 Lytic activity of $M1_{58-66}$- and $NP_{383-391}$-specific $CD8^+$ T cells against target cells transfected with various M1-NP-eGFP encoding plasmids

A) Gating strategy used to assess the number of viable $eGFP^+$ target cells. First dotplot demonstrates a gate for the transfected target cells, second gate demonstrates the viable cells and the third gate demonstrates the $eGFP^+$ cells. B) Percentage lytic activity exerted by the $M1_{58-66}$-specific $CD8^+$ T cell clone. C) Percentage lytic activity exerted by the $NP_{383-391}$-specific $CD8^+$ T cell clone. Target cells were transfected with chimeric M1-NP-eGFP fusion plasmids that encode the M1 protein of the avian A/H5N1 virus (WT avian; purple squares), the M1 protein of the human A/H3N2 virus (WT human; blue squares), the M1 protein of avian A/H5N1 virus with extra-epitopic amino acid residues of the human A/H3N2 virus (A→H ABCDE; purple circles) and the M1 protein of human A/H3N2 virus with extra-epitopic amino acid residues of the avian A/H5N1 virus (H→ A ABCDE; blue circles). Data points represent the mean and error bars indicate the standard deviation (SD) of quadruplicates (n=4). ** Indicates that all groups were statistically significantly different from each other after correction for multiple hypothesis testing using a false discovery rate (FDR) of 0.01. * Indicates that only the A→H ABCDE group was significantly lower.

Differential activation of $M1_{58-66}$-specific $CD8^+$ T cells by M1 protein derived from avian or human IAV

Next, we investigated activation of $M1_{58-66}$-specific $CD8^+$ T cells after stimulation with HLA-A*0201/B*2705 positive EBV transformed B cells expressing the respective chimeric M1-NP-eGFP fusion proteins. Upon stimulation, the expression of the activation markers CD137, CD69 and CD107a by $M1_{58-66}$- and $NP_{383-391}$-specific $CD8^+$ T cells was determined by flow cytometry (Fig. 4A). Because both the E:T ratio and incubation time had to be adapted for this purpose, we also assessed the lytic activity of the $CD8^+$ T cells in a FATT-CTL assay under these conditions (Fig. 4). Again, only the $M1_{58-66}$-specific $CD8^+$ T cells displayed differential lytic activity against target cells expressing the M1 protein derived from avian or human IAV. Once again, the reciprocal exchange of the extra-epitopic amino acid residues partially reversed the lytic activity pattern (Fig. 4B). The lytic activity of the $NP_{383-391}$-specific $CD8^+$ T cells to the respective M1 proteins was similar for all chimeric M1-NP-eGFP fusion proteins (Fig. 4B).

Upon stimulation with the M1 protein derived from avian IAV A/Vietnam/1194/2004 (H5N1), a significantly higher percentage (approximately 2.5 fold) of $M1_{58-66}$-specific $CD8^+$ T cells was positive for the activation markers than after stimulation with the M1 protein derived from the human influenza virus A/Netherlands/018/1994 (H3N2) (CD137: 19% vs 7,6%; CD69: 2,5% vs 1,1% and CD107a: 54% vs 20% respectively) (Fig. 4C). Such differences were not observed for the $NP_{383-391}$-specific $CD8^+$ T cells, although CD107a expression was slightly higher after stimulation with M1 protein from the avian virus (1.1 fold), but this difference was far smaller than that observed for the $M1_{58-66}$-specific $CD8^+$ T cells (Fig. 4D). Again, the reciprocal exchange of the extra-epitopic amino acid residues partially reversed the pattern of differential activation of the $M1_{58-66}$-specific $CD8^+$ T cells. The introduction of extra-epitopic amino acid residues from the human IAV into the M1 protein of the avian IAV reduced activation of the $M1_{58-66}$-specific $CD8^+$ T cells and vice versa (Fig. 4C). The exchange of amino acid residues in the M1 protein resulted in minor differences in activation of the $NP_{383-391}$-specific $CD8^+$ T cells. Although some of these small differences were statistically significant, they did not correlate with the activation pattern observed for the $M1_{58-66}$-specific $CD8^+$ T cells (Fig. 4D). In addition to assessing the expression of CD107a, a proxy for degranulation and lytic activity, we also assessed IFN-γ production by the $CD8^+$ T cells as an alternative functional property of $CD8^+$ T cell activation by ELISpot assay. Again, stimulation with the M1 protein derived from the avian IAV resulted in a significantly higher number of IFN-γ producing $M1_{58-66}$-specific $CD8^+$ T cells than after stimulation with the M1 protein of human IAV (228 vs 54 IFN-γ^+ spots/10^4 cells) (Fig. 5A). No significant difference was observed for the $NP_{383-391}$-specific $CD8^+$ T cells (Fig. 5B). Thus a good correlation was observed between the differential expression of activation markers, including CD107a, lytic activity and IFN-γ production by $M1_{58-66}$-specific $CD8^+$ T cells, which was

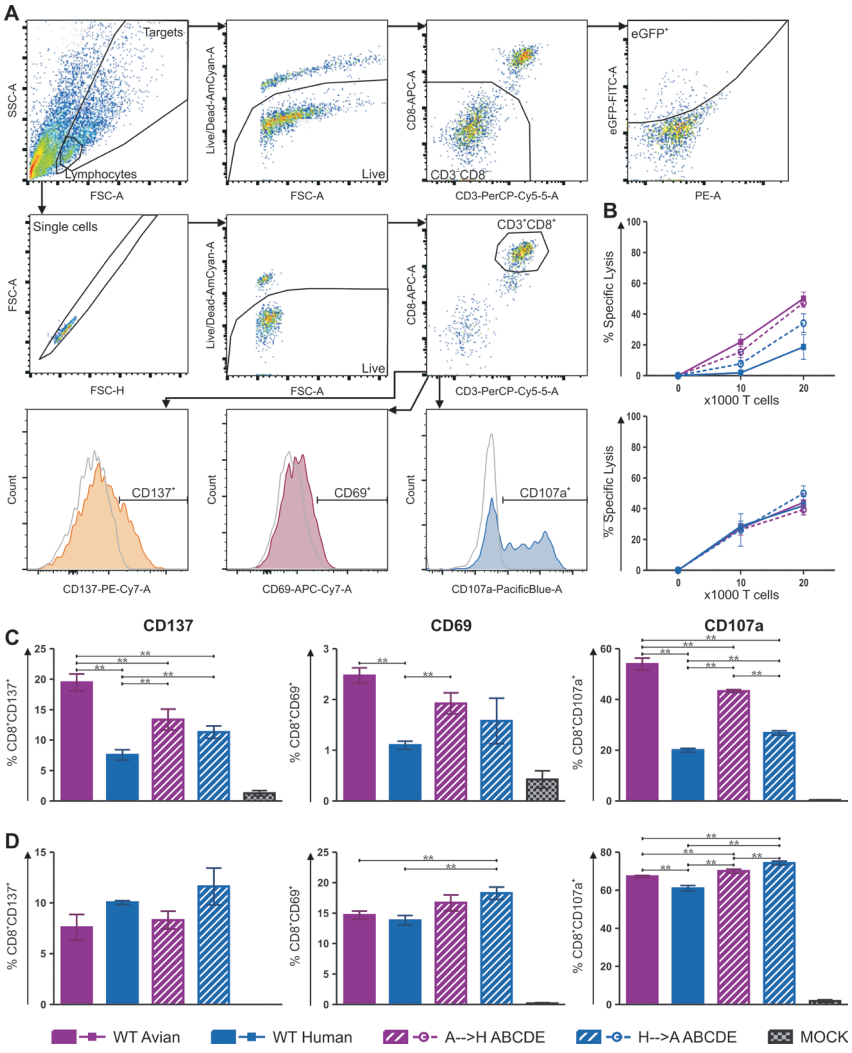

Figure 4 Activation of $M1_{58-66}$- and $NP_{383-391}$-specific $CD8^+$ T cells after stimulation with target cells transfected with various M1-NP-eGFP encoding plasmids

A) Dot plots on top from left to right gate the transfected target cells, the viable cells, the $CD3^-CD8^-$ cells and finally the $eGFP^+$ cells. This gating was used to assess the lytic activity of the $CD8^+$ T cell clone in a FATT-CTL dependent manner. Lower dot plots gate the lymphocytes, single cells, viable cells, $CD3^+CD8^+$ cells followed by gating for the upregulation of activation markers CD137, CD69 or CD107a after stimulation with target cells transfected with the M1-NP-eGFP plasmids (colored histograms) or the eGFP only plasmid (gray histogram). B) Percentage lytic activity exerted by the $M1_{58-66}$-specific $CD8^+$ T cells (top) and $NP_{383-391}$-specific $CD8^+$ T cells (bottom). Upregulation of activation markers CD137, CD69 and CD107a on $M1_{58-66}$-specific $CD8^+$ T cells (C) or the $NP_{383-391}$-specific $CD8^+$ T cells (D) after stimulation with target cells transfected with M1-NP-eGFP plasmids that encode the M1 protein of the avian A/H5N1 virus (WT avian; purple squares / purple filled bars), the M1 protein of the human A/H3N2 virus (WT human; blue squares / blue filled bars), the avian A/H5N1 M1 protein with extra-epitopic amino acid residues of the human A/H3N2 virus (A→H ABCDE; purple circles / purple striped bars), the human A/H3N2 M1 protein with extra-epitopic amino acid residues of the avian A/H5N1 virus (H→A ABCDE; blue circles / blue striped bars) or eGFP only (Mock; dashed bar). Data points represent the mean and error bars indicate the standard deviation (SD) of quadruplicates (n=4). ** Indicates statistically significant differences between groups after correction for multiple hypothesis testing using a FDR of 0.01.

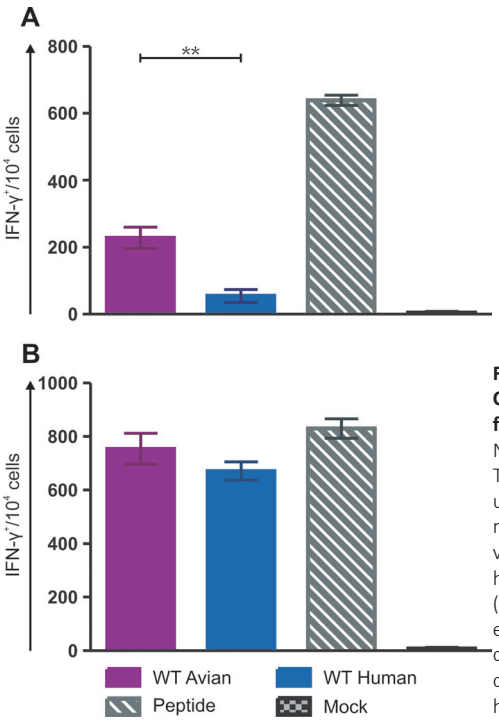

Figure 5 IFN-γ response by $M1_{58-66}$- and $NP_{383-391}$-specific $CD8^+$ T cells after stimulation with target cells transfected with various M1-NP-eGFP encoding plasmids
Number of IFN-γ positive spots/10^4 $M1_{58-66}$-specific $CD8^+$ T cells (A) or $NP_{383-391}$- specific $CD8^+$ T cells (B) after stimulation by target cells transfected with M1-NP-eGFP plasmids that encode the M1 protein of the avian A/H5N1 virus (WT avian; purple bars) or the M1 protein of the human A/H3N2 virus (WT human; blue bars) or eGFP only (Mock; dashed bar). Data points represent the mean and error bars indicate the standard deviation (SD) of quadruplicates (n=4). ** Indicates a statistically significant difference between groups after correction for multiple hypothesis testing using a FDR of 0.01.

dependent on the source of the M1 proteins used for stimulation and their extra-epitopic amino acid residues.

The M1 protein context determines the kinetics of $M1_{58-66}$-specific $CD8^+$ T cell activation after stimulation with virus-infected cells

Finally, we wished to assess whether recognition of cells, infected with IAVs carrying either of the respective M1 proteins, could lead to differential activation of $M1_{58-66}$-specific $CD8^+$ T cells. To this end, isogenic recombinant viruses, containing the matrix (M) gene segment of avian virus A/Vietnam/1194/2004 (H5N1) or human virus A/Netherlands/178/1995 (H3N2), were used to infect A549-HLA-A*0201$^+$ target cells. Two hours post inoculation (p.i.) these infected target cells were incubated with the $M1_{58-66}$-specific $CD8^+$ T cells and the kinetics of CD137, CD69 and CD107a expression was assessed (Fig. 6A).

As shown in figure 6, stimulation with virus containing the M gene segment of human influenza virus A/Netherlands/178/1995 (H3N2) resulted in delayed activation of $M1_{58-66}$-specific $CD8^+$ T cells compared to stimulation with virus containing the M gene segment of avian influenza virus A/Vietnam/1194/2004 (H5N1). One of the earliest markers of T cell activation was expression of the degranulation marker CD107a. Upon stimulation with peptide-pulsed A549-

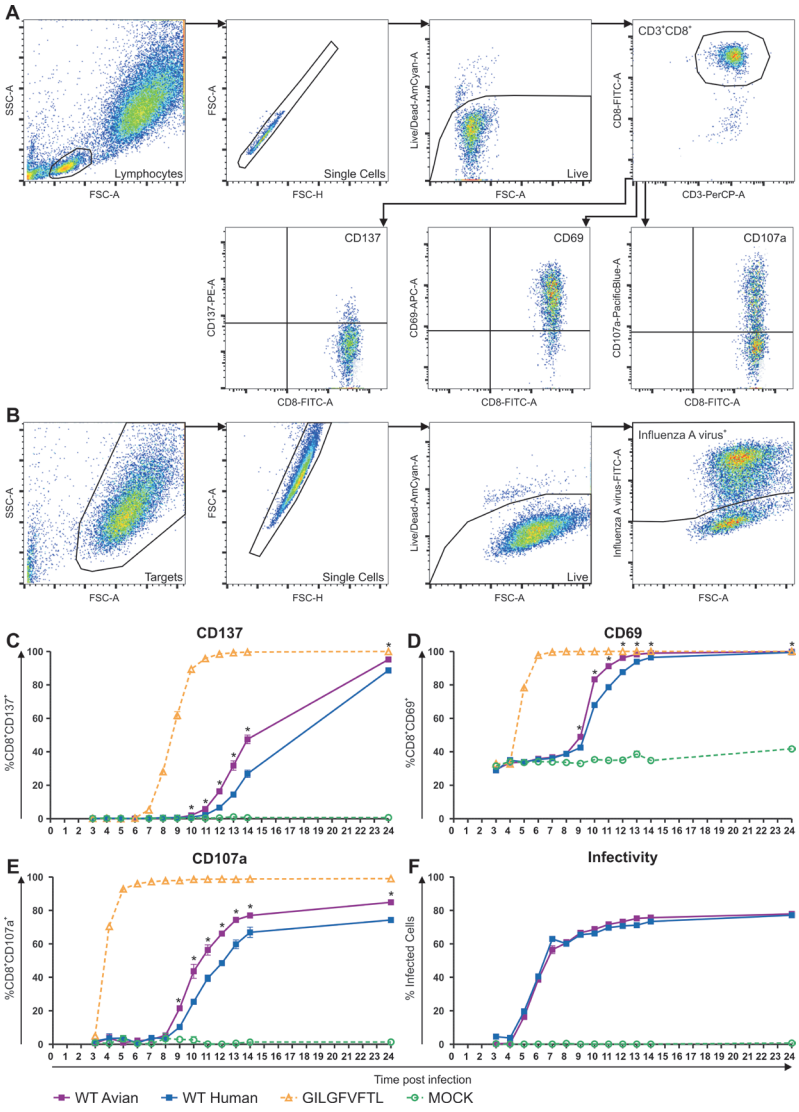

Figure 6 Activation kinetics of $M1_{58-66}$-specific $CD8^+$ T cells after stimulation with cells infected with isogenic influenza A viruses with gene segment 7 of human or avian influenza A viruses

A) Gating strategy used to assess the upregulation of activation markers on $M1_{58-66}$-specific $CD8^+$ T cells. Dot plots gate the lymphocytes, single cells, viable cells, $CD3^+CD8^+$ cells followed by gating for the upregulation of activation markers CD137, CD69 or CD107a. B) Gating strategy used to determine infection efficiency of the target cells. Dot plots gate the target cells, the viable cells and finally the influenza A virus$^+$ cells. Expression of activation markers CD137 (C), CD69 (D) and CD107a (E) by $M1_{58-66}$-specific $CD8^+$ T cells after stimulation with A549-HLA-A*0201$^+$ cells infected with recombinant virus A/PuertoRico/8/1934 with gene segment 7 of avian virus A/Vietnam/1194/2004 (H5N1) (WT avian; purple squares) or human virus A/Netherlands/178/1995 (H3N2) (WT human; blue squares) or pulsed with $M1_{58-66}$ peptide (GILGFVFTL; yellow triangles) or untreated (MOCK; green circles). F) Percentage infected A549-HLA-A*0201$^+$ cells at each time point (without T cells). X-axis represent hours post infection. A549-HLA-A*0201$^+$ cells were infected/peptide pulsed for 1 hr and then used to stimulate $M1_{58-66}$-specific $CD8^+$ T cells. Data points represent the mean and error bars indicate the standard deviation (SD) of triplicates (n=3). * Indicates statistically significant differences between avian and human derived viruses after correction for multiple hypothesis testing using a FDR of 0.01.

HLA*A0201$^+$ cells, $M1_{58-66}$-specific CD8$^+$ T cells degranulated almost immediately. After stimulation with virus-infected cells, CD107a expression was detected as early as 9 hours p.i.. Similar observations were made for CD137 and CD69, although CD137 expression started at a later time point than CD69 and CD107a. In any case, the proportion of CD8$^+$ T cells that became activated and expressed either activation marker was significantly higher after stimulation with the virus containing the M gene segment of avian A/H5N1 at every time point p.i. (highest fold difference for CD137 at 14 hours p.i. (1.8 fold), for CD107a and CD69 at 10 hours p.i. 1.7 and 1.2 fold respectively) (Fig. 6C,D,E). The replication kinetics of both viruses was very similar and resulted in equal numbers of infected target cells, which excluded infection rates as the cause of the differences in the kinetics of CD8$^+$ T cell activation (Fig. 6B,F). These results clearly indicate that extra-epitopic amino acid residues of the $M1_{58-66}$ epitope affect the recognition of viruses containing the M gene segment of the seasonal A/H3N2 virus by $M1_{58-66}$-specific CTLs.

Evolution of extra-epitopic amino acid residues of the $M1_{58-66}$ epitope

In order to link immunologic observations with the epidemiology of IAVs that circulated in the human population we examined the origin of gene segment 7, which encodes the M1 protein, and the evolution of amino acid residues outside the $M1_{58-66}$ epitope. The M1 protein in the human population originates from the 1918 pandemic virus (Fig. 1) [81-83, 453, 454]. Of interest, the extra-epitopic substitutions described in this paper were present in most human IAVs isolated since 1918 and were maintained over 100 years of viral evolution in the human population (Fig. 7; Table 1). The only exception was the V115I substitution which was not observed in the only 1918 virus sequence (A/BrevigMission/1/1918) available in the influenza virus resource database (Fig. 7; Table 1). Interestingly, the H1N1 IAVs that caused the pandemic in 2009 (H1N1pdm09) and that replaced the old seasonal H1N1 viruses possess a M1 protein of avian/swine signature (Fig. 1; Fig. 7; Table 1).

The extra-epitopic amino acid residues of human IAVs were occasionally observed in avian and swine IAVs, although they were mainly present in isolation. The avian variants of these extra-epitopic amino acid residues were observed with a higher frequency in both avian and swine IAVs (Fig. 7; Table 1). Avian variants of these extra-epitopic amino acid residues were rarely observed in human IAVs (Fig. 7; Table 1). The arginine residue at position 27 (present in human viruses) was the exception as it was also observed with a high frequency in avian and swine viruses (77,4% and 99,6% respectively) (Fig. 7; Table 1).

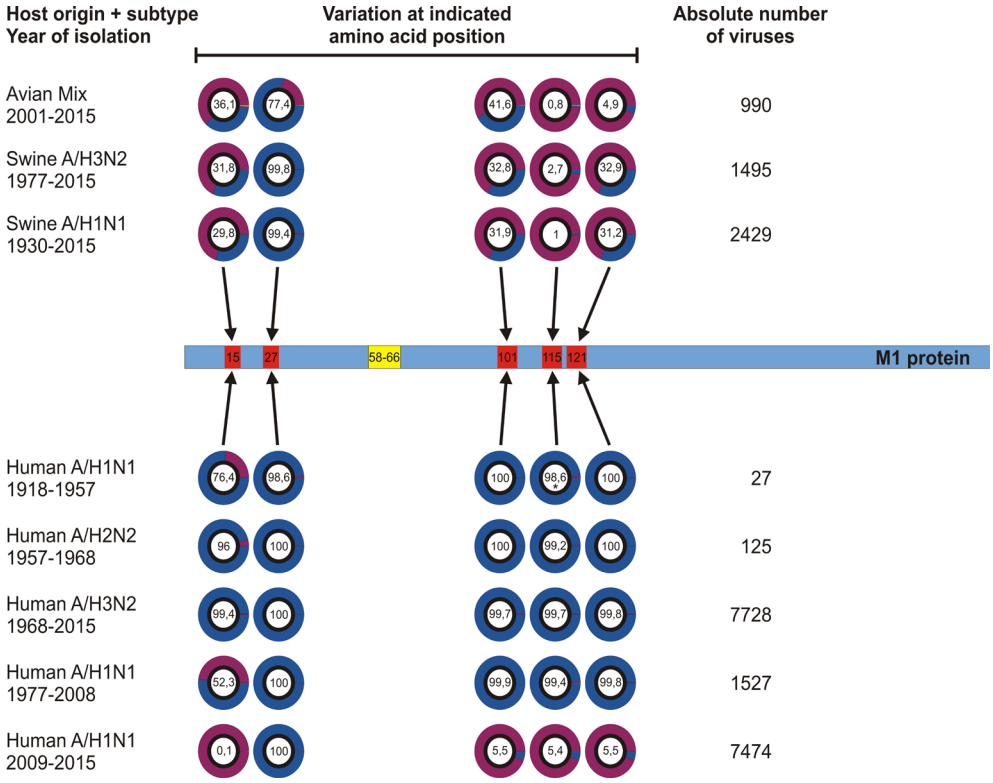

Figure 7 Frequency amino acid variations region flanking $M1_{58-66}$
Frequency of amino acid variations at positions 15, 27, 101, 115 and 121 in the M1 protein of avian, swine and human influenza A viruses isolated in the indicated time period. Frequencies were based on the total number of M1 protein sequences present in the the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genomes/FLU) database at April 11th 2015.
Pie charts represent frequency of avian (purple) and human (blue) amino acid residues based on the variations observed in figure 2. Frequency of other amino acid residues at these positions are indicated in orange. Frequencies of the preferred human IAV amino acid residues are indicated in the pie charts. * Only one 1918 sequence (A/BrevigMission/1/1918) could be obtained from the NCBI database, which was the only virus in this group containing the 115V residue, the following H1N1 viruses from this group were from the 1930s. A more detailed overview of the frequencies of the respective amino acid variations at these positions can be found in Table 1.

Discussion

In the present study, we demonstrated that extra-epitopic amino acid residues affect $CD8^+$ T cell recognition of the highly conserved immunodominant HLA-A*0201 restricted $M1_{58-66}$ IAV epitope. Naturally occurring amino acid variation at positions outside the epitope contributed to the observed differences in epitope recognition of avian and human IAVs. The origin from which the M1 protein was derived, an avian or human IAV, determined the kinetics of $CD8^+$ T cell activation after stimulation with virus-infected cells. Recognition of the M1 protein derived from human IAV delayed and impaired the activation and reduced lytic activity of the

Table 1 Frequency amino acid difference region flanking M1$_{58-66}$ epitope

Virus†				15*			27*			95*			101*			115*			121*		
Species	Serotype	Year	Total	aa	%	#	aa	%	#	aa	%	#	aa	%	#	aa	%	#	aa	%	#
Avian	Mix	2001 - 2015	990	**I**	**62,7**	**621**	R	77,4	766	R	78,8	780	**K**	**58**	**574**	**V**	**98,5**	**975**	**T**	**94,8**	**939**
				V	36,1	357	**K**	**22,1**	**219**	K	20,6	204	R	41,6	412	I	0,8	8	A	4,9	49
				T	0,6	6	X	0,2	2	X	0,4	4	X	0,2	2	M	0,3	3	P	0,1	1
				X	0,2	2	T	0,1	1	T	0,1	1	T	0,1	1	L	0,1	1	N	0,1	1
				Y	0,1	1	G	0,1	1	S	0,1	1	G	0,1	1	G	0,1	1			
				F	0,1	1	S	0,1	1							F	0,1	1			
				L	0,1	1															
Swine	A/H3N2	1977 - 2015	1495	**I**	**67,9**	**1015**	R	99,8	1492	R	78,8	780	**K**	**67,2**	**1004**	**V**	**97,2**	**1453**	**T**	**67,1**	**1003**
				V	31,8	475	**K**	**0,2**	**3**	K	20,6	204	R	32,8	491	I	2,7	40	A	32,9	492
				T	0,1	2				X	0,4	4				X	0,1	1			
				L	0,1	2				T	0,1	1				T	0,1	1			
				X	0,06	1				S	0,1	1									
Swine	A/H1N1	1930 - 2015	2429	**I**	**70**	**1700**	R	99,4	2415	R	68,3	1658	**K**	**68**	**1652**	**V**	**99**	**2405**	**T**	**68,8**	**1670**
				V	29,8	724	**K**	**0,6**	**14**	K	31,7	771	R	31,9	776	I	1	24	A	31,2	758
				T	0,2	4							N	0,04	1				P	0,04	1
				M	0,04	1															
Human	A/H1N1	1918 - 1957	72	**V**	**76,4**	**55**	**R**	**98,6**	**71**	R	63,9	46	**R**	**100**	**72**	**I**	**98,6**	**71**	**A**	**100**	**72**
				I	23,6	17	K	1,4	1	K	36,1	26				V¶	1,4	1			
Human	A/H2N2	1957 - 1968	125	**V**	**96**	**120**	**R**	**100**	**125**	R	100	125	**R**	**100**	**125**	**I**	**99,2**	**124**	**A**	**100**	**125**
				I	4	5										V	0,8	1			
Human	A/H3N2	1968 - 2015	7728	**V**	**99,4**	**7682**	**R**	**100**	**7728**	**K§**	**89,9**	**6944**	**R**	**99,7**	**7705**	**I**	**99,7**	**7701**	**A**	**99,8**	**7709**
				I	0,6	45				**R**	**10,1**	**783**	K	0,3	21	V	0,3	25	T	0,2	19
				X	0,01	1				X	0,01	1	T	0,03	2	X	0,01	1			
																M	0,01	1			
Human	A/H1N1	1977 - 2008	1527	**V**	**52,3**	**798**	**R**	**100**	**1527**	R	98,8	1509	**R**	**99,9**	**1526**	**I**	**99,4**	**1518**	**A**	**99,8**	**1524**
				I	47,7	728				K	1,2	18	K	0,1	1	V	0,5	8	T	0,2	3
				A	0,1	1										M	0,1	1			
Human	A/H1N1	2009 - 2015	7474	I	99,8	7461	**R**	**100**	**7474**	R	99,8	7460	K	94,5	7063	V	94,5	7064	T	94,5	7063
				V	**0,1**	**10**				K	0,2	14	**R**	**5,5**	**410**	**I**	**5,4**	**406**	**A**	**5,5**	**411**
				T	0,03	2							E	0,01	1	M	0,1	4			
				M	0,01	1															

† All amino acid sequences present in the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genomes/FLU) database at April 11th 2015.

* Position in the M1 amino acid sequence; Year = years of isolation that were present in the database; aa = amino acid; % = frequency; # = absolute number of viruses.

Indicated in **bold** are the amino acids present in the avian H5N1 (A/Vietnam/1194/04) and human H3N2 (A/Netherlands/018/1994) used in the present study.

§ the 95K mutation was introduced in 1997 and rapidly fixated in human seasonal A/H3N2 viruses in the following years; ¶ Only one 1918 sequence (A/BrevigMission/1/1918) could be obtained from the NCBI database, which was the only virus in this group containing the 115V residue, the following H1N1 viruses from this group were from the 1930s.

$M1_{58-66}$-specific $CD8^+$ T cells compared to recognition of M1 protein derived from an avian IAV. This difference in recognition may have implications for virus clearance in HLA-A*0201 individuals and spread of the IAVs in the human population. The differential recognition of the $M1_{58-66}$ epitope may explain in part previously described differences in recognition of human seasonal A/H3N2 virus, avian A/H5N1 and A/H7N9 viruses [316, 536].

Although, it is not fully clear what the selective pressure is for the preferred use of the amino acid residues under investigation, it is tempting to speculate that evasion from recognition by $M1_{58-66}$-specific $CD8^+$ T cells plays a role. It has been suggested that the immunodominance of the epitope serves as a stealth strategy and that impaired function of $M1_{58-66}$-specific $CD8^+$ T cells explained the virus capacity to evade recognition by these T cells [625]. However, this is a matter of debate [626] also because HLA-A*0201 positive individuals display stronger CTL responses after IAV infection [313].

Most likely, extra-epitopic amino acid residues affect the processing and presentation of the $M1_{58-66}$ epitope. Differences in translocation by TAP [413, 414, 627] or trimming of peptides by ER resident proteases like ER amino peptidase 1 or 2 [628], may not have contributed, because TAP typically transports peptides of 8-16 amino acids long [414] and the amino acid substitutions under investigation are too distantly located from the epitope to be able to have an effect on these processes. More likely, earlier steps in the antigen processing pathway are involved, like degradation by the proteasome [629]. However, because the constitutive proteasome potentially cleaves inside the epitope sequence [630], alternative proteases like the immunoproteasome [631] or non-proteasomal proteases [632], like tripeptidypeptidase (TPPII) [633, 634], are more likely candidates.
Based on these studies we hypothesize that the difference in extra-epitopic amino acid residues either change the cleavage pattern of the M1 protein and/or define which protease processes the M1 protein, which will eventually determine the extent of $M1_{58-66}$ epitope presentation.

It has been shown that amino acid residues flanking a mouse CTL epitope altered recognition of IAV [591, 594, 595, 635, 636]. However, these findings were obtained with artificially introduced mutations. In the present study, we show for the first time that naturally occurring variation at positions outside the epitope influences antigen processing resulting in differential $CD8^+$ T cell recognition of human and avian IAVs. Of note, mutations flanking CTL epitopes affecting $CD8^+$ T cell recognition have been observed in viruses causing chronic infections [418, 419, 615]. In most cases, these mutations were located in close proximity to the epitope (within 10 amino acids). In contrast, the extra-epitopic variation in amino acid residues observed in the present

study were more distant from the epitope (over 30 amino acids), and to the best of our knowledge this has not been observed previously. Since the reciprocal exchange of the five extra-epitopic amino acid residues described in this study only partly reverse the $CD8^+$ T cell recognition patterns between the M1 protein of the avian and human IAV, it cannot be excluded that other substitutions more distant from the $M1_{58-66}$ epitope, e.g. at positions 137, 166, 167, 168, 207, 218, 224, 230 and 232 (Fig. 2), also contribute to antigen processing and thus to differential recognition.

Analysis of the extra-epitopic residues in all M1 protein amino acid sequences of avian human and swine IAVs available in the influenza virus resource database [http://www.ncbi.nlm.nih.gov/genomes/FLU] revealed that 15V, 27R, 101R, 115I and 121A were the preferred residues in human IAVs (Fig. 7; Table 1). Residues 15I, 101K, 115V and 121T, were preferred in avian IAVs. However, residue 27K was a minor variant in avian and swine IAVs (Fig. 7; Table 1). It would be of interest to determine the minimal set of amino acid residues that are responsible for the observed differences in recognition. Of note, within the 60 amino acid distance from the $M1_{58-66}$ epitope the R95K substitution was rapidly fixed in human A/H3N2 viruses after its initial introduction in 1997 (Table 1). Its rapid fixation suggests that this substitution might also contribute to evasion from recognition by $M1_{58-66}$-specific $CD8^+$ T cells. It has been hypothesized that the preferred avian or human IAV amino acid residues at position 115 and 121 of the M1 protein reflect viral host adaptation [453]. However, as the H1N1pdm09 contained a M1 protein with the preferred avian/swine amino acid residue at these positions this might not be the case (Fig. 1; Fig. 7; Table 1) [84]. Evasion from recognition by $M1_{58-66}$-specific CTLs, as demonstrated in the present study, may provide an alternative explanation. It would therefore be of interest to monitor acquisition of these preferred human amino-acid residues in the M1 amino acid sequence of H1N1pdm09 IAVs.

The pandemic of 2009 demonstrated that the frequency of pre-existing IAV-specific $CD8^+$ T cells inversely correlated with disease severity [344, 612]. Compared to the pandemics of 1918, 1957 and 1968, the pandemic of 2009 was generally considered milder [94, 102]. Especially in the elderly morbidity and mortality was relatively low [102]. This was attributed to the presence of antibodies in this age group induced by infection with A/H1N1 viruses that circulated prior to 1957 and that are antigenically related to the H1N1pdm09 virus [103, 241]. In contrast to previous pandemic viruses which possess a M1 protein of human signature, the H1N1pdm09 virus possessed a M1 protein of avian/swine signature (Fig. 1; Fig. 7; Table 1). Consequently, our data suggest that in previously infected HLA-A*0201 positive individuals, the 2009 pandemic viruses were better recognized by preexisting $M1_{58-66}$-specific $CD8^+$ T cells, which also contributed to protective immunity.
The continuous pandemic threat posed by avian IAVs of various subtypes and the

emergence of drift variants of seasonal IAVs underscores the need for vaccines that could induce broad protective immunity, so called universal vaccines. IAV-specific $CD8^+$ T cells are predominantly directed to conserved epitopes, including $M1_{58-66}$, and are considered an important correlate of cross-protective immunity [316, 344, 528, 536, 611-613]. Therefore, universal influenza vaccines should aim at the induction of virus-specific CTL responses. Although the extent of exerting antiviral activity of the $M1_{58-66}$-specific $CD8^+$ T cells during subsequent influenza virus infections will depend on the origin of the virus (avian or human), the present study suggests that for the efficient induction of CTL responses proteins of avian IAVs may be advantageous over those derived from human IAVs. This may also apply to live attenuated influenza vaccines that are known to induce CTL responses [495, 560]. These responses may be improved with the use of viral proteins originating from avian IAVs. Alternatively, vaccine approaches that circumvent the antigen processing pathways are of interest [627, 637].

Collectively, we have demonstrated that the conserved $M1_{58-66}$ epitope is differentially recognized by epitope-specific $CD8^+$ T cells depending on the origin of the M1 protein. Extra-epitopic amino acid residues are responsible for the differential recognition, which indicates that differences in antigen processing and presentation are at the basis of these observations. In the context of a M1 protein of human signature the epitope is relatively poorly recognized compared to the M1 protein of avian viruses. It can be speculated that the possession of a M1 protein of human signature offers the virus an advantage by impairing recognition by specific $CD8^+$ T cells. Consequently, these viruses may replicate better in HLA-A*0201 positive individuals. Since HLA-A*0201 has a high prevalence in the human population, this also may impact the spread of the virus in the human population. In addition, our findings may have implications for the development of vaccines that aim at the induction of virus-specific CTL responses.

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CHAPTER 6:

Novel G3/DT adjuvant promotes the induction of protective T cells responses after vaccination with a seasonal trivalent inactivated split-virion influenza vaccine

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Abstract

Vaccines used against seasonal influenza are poorly effective against influenza A viruses of novel subtypes that may have pandemic potential. Furthermore, pre(pandemic) influenza vaccines are poorly immunogenic, which can be overcome by the use of adjuvants. A limited number of adjuvants has been approved for use in humans, however there is a need for alternative safe and effective adjuvants that can enhance the immunogenicity of influenza vaccines and that promote the induction of broad-protective T cell responses. Here we evaluated a novel nanoparticle, G3, as an adjuvant for a seasonal trivalent inactivated influenza vaccine in a mouse model. The G3 adjuvant was formulated with or without steviol glycosides (DT, for diterpenoid). The use of both formulations enhanced the virus-specific antibody response to all three vaccine strains considerably. The adjuvants were well tolerated without any signs of discomfort. To assess the protective potential of the vaccine-induced immune responses, an antigenically distinct influenza virus strain, A/Puerto Rico/8/34 (A/PR/8/34), was used for challenge infection. The vaccine-induced antibodies did not cross-react with strain A/PR/8/34 in HI and VN assays. However, mice immunized with the G3/DT-adjuvanted vaccine were partially protected against A/PR/8/34 infection, which correlated with the induction of anamnestic virus-specific $CD8^+$ T cell responses, which were not observed with the use of G3 without DT. Both formulations induced maturation of human dendritic cells and promoted antigen presentation to a similar extent. In conclusion, G3/DT is a promising adjuvant formulation that not only potentiates the antibody response induced by influenza vaccines, but also induces T cell immunity which could afford broader protection against antigenically distinct influenza viruses.

Introduction

Influenza virus infections cause excess morbidity and mortality in the human population [65]. For the prevention of severe disease and mortality it is recommended to vaccinate high-risk groups against influenza annually. Commonly, inactivated trivalent influenza vaccines are used that elicit strain-specific antibodies [476, 493]. However, these vaccines fail to afford life-long protection, mainly due to antigenic drift of influenza viruses [68, 69, 79, 400]. Influenza vaccine strains need to be updated almost annually, in order to match circulating influenza strains as closely as possible [478]. A mismatch of vaccine strains may lead to reduced effectiveness of influenza vaccines [479, 480]. Furthermore, the emergence of an antigenically distinct influenza virus of a novel subtype, may trigger an influenza pandemic to which inactivated seasonal influenza vaccines afford little protection [79]. Ideally, vaccine formulations are used that not only induce neutralizing antibodies to seasonal influenza viruses but also induce more broadly protective immune responses.

Strain-specific virus neutralizing antibodies are mainly directed to the variable globular head region of the viral hemagglutinin (HA) [68, 236], although cross-reactive antibodies have been identified [244, 251, 254, 257, 266]. In addition, virus-specific $CD4^+$ T cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) contribute to protective immunity. CTLs, which are directed to conserved internal proteins predominantly, contribute to heterosubtypic immunity [311, 315-317, 337-339, 534-536, 538, 539, 638, 639]. The main function of CTLs is to recognize and eliminate virus infected cells, thus restricting virus replication and accelerating viral clearance. The existence of cross-reactive CTLs induced by previous infections and their role in protection against heterologous influenza virus strains in humans was demonstrated in several studies [316, 317, 344-346, 535, 536, 541]. Currently used inactivated influenza vaccines inefficiently elicit cross-reactive CTL responses [495, 640], which may be related to their inability to deliver proteins into the cytosol of antigen presenting cells (APCs) for endogenous antigen processing and MHC class I presentation. Therefore, there is considerable interest in adjuvants and antigen delivery systems that not only improve antibody responses of current (inactivated) influenza vaccines, but that also induce cross-reactive CTLs. It has been demonstrated that immune stimulating complex (ISCOM) based vaccines induce strong antibody responses as well as virus-specific CTL responses both in animal models and humans [275, 276, 513, 514, 641-645], whereas current adjuvants, like aluminum salts ($Al(OH)_3$ and $AlPO_3$) and oil-in-water adjuvants (e.g. MF59, ASO3 and Freund's adjuvant) enhance antibody responses, but fail to induce CTL responses [501-503, 641]. Most likely, ISCOMs facilitate delivery of viral proteins into the cytosol of APCs where protein degradation and liberation of antigenic peptides by the proteasome takes place [622, 646].

In the present study, we evaluated G3, a novel nanoparticle adjuvant as an alternative for ISCOMs. In contrast to ISCOMs, G3 lacks the phospholipid component, is easy to produce, allows incorporating molecules of various kinds, like additional adjuvant components or antigens, and further reduces possible side effects associated with the use of ISCOMs. In this study, the G3 adjuvant was formulated with or without steviol glycosides (deterpenoids (DT); G3/DT), which are known for their ability to promote lymphocyte responses [647]. We evaluated these novel G3 adjuvants for their capacity to enhance the immunogenicity of a commercially available seasonal trivalent inactivated split virion influenza vaccine in a mouse model. It was concluded that G3/DT is a versatile adjuvant system that not only enhances the antibody responses, but that also induces cross-reactive virus-specific $CD8^+$ T cell responses.

Materials and Methods

Adjuvants

G3 formulations were prepared with quillaja saponins isolated from the bark of *Quillaja saponaria Molina* and supplied as QS21 (Desert King CF, USA). In contrast to the preparation of conventional 40nm ISCOM particles, G3 is produced without phosphatidylcholine, resulting in a smaller spherical particle size of 20nm (data not shown). The G3/DT adjuvant preparation differs from the G3 preparation by the incorporation of DT. DT was isolated from a water extraction of *Rebaudiana Bertoni* (Prodalysa, Concón, Chile) from which steviol glycosides were purified by membrane purification carried out with ultrafiltration and nanofiltration membranes and water eluted ion exchange to obtain a purity of >95%. The molar ratio quillaja saponin (a triterpen) and DT was 2:1. The adjuvant dose for immunization of mice was 5µg G3 or G3/DT based on quillaja saponin content.

Vaccine preparation

A commercially available seasonal trivalent split virion vaccine of the 2012/2013 influenza season (VAXIGRIP®) (2012/2013, Sanofi Pasteur MSD, Brussels, Belgium) was used in the present study and contained components of the vaccine strains: NYMC X-179A derived from strain A/California/7/2009 (H1N1pdm09), IVR-165 derived from strain A/Victoria/361/2011 (H3N2) and NYMC BX-39 derived from B/Hubei-Wujiang/158/2009, a B/Wisconsin/1/2010 like virus. The vaccine dose used in mice contained 5µg hemagglutinin of each vaccine strain.

Influenza virus

Influenza viruses A/PR/8/34 (H1N1), X181 (derived from A/California/7/2009), IVR-165 and NYMC BX-39 were propagated in embryonated chicken eggs as described previously [338]. Infectious virus titers were determined in Madin-Darby Canine Kidney (MDCK) cells as described previously [545].

Immunization and inoculation of mice

Specified pathogen free, 6-8 weeks old female C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Mice received two subcutaneous (s.c.) immunizations in the dorsal neck region in a total volume of 200µl with an interval of four weeks. Mice (n=7-14 per group) received 5µg HA of each of the vaccine strains in the trivalent split virion vaccine with 5µg G3/DT (Group 1), G3 (Group 2) or without adjuvant (Group 3). Control mice received phosphate buffered saline (PBS) with 5µg G3/DT (Group 4), G3 (Group 5) or without adjuvant (Group 6 and 7). Four weeks after the second immunization mice were inoculated intranasally (i.n.) with a lethal dose (5.10^4 $TCID_{50}$) of influenza virus A/PR/8/34 in a volume of 50µl (Group 1- 6). Group 7 received 50µl of PBS i.n. (Table 1). Clinical signs, including weight loss, were monitored after vaccination and during the infection. Mice were euthanized on day 4 (n=8 for group 1-6, n=4 for group 7) and day 6 post inoculation (p.i.) (n=6 for group 1-6, n=3 for group 7) or when they displayed weight loss of >25% (humane endpoint, mice were scored as dead). Mice were bled via orbital puncture and lungs and spleens were resected. Blood was collected via submandibular bleed just before the first and second vaccination and before challenge infection. All biotechnical procedures were performed under anesthesia with 4% isoflurane in O_2. Animals were housed in individually ventilated cages (IVC-units) and had access to food and water ad libitum. An independent animal ethics committee (DEC consult) approved the experimental protocol before start of the experiment.

Table 1 Experimental groups and design of the study

Experimental group	Vaccination			A/PR/8/34 challenge
	Split virion vaccine	Adjuvant G3/DT	Adjuvant G3	
1	+	+	–	+
2	+	–	+	+
3	+	–	–	+
4	–	+	–	+
5	–	–	+	+
6	–	–	–	+
7	–	–	–	–

Serology

Serum samples were obtained before and twenty-eight days after the first and twenty-eight days after the second vaccination as well as on day 4 or 6 p.i. and were stored at -20°C until use. Sera were tested for the presence of antibodies to either influenza virus A/PR/8/34, IVR-165, X181 or BX-39 using a hemagglutination inhibition (HI) assay as described previously [648]. Post infection ferret sera, raised against the influenza viruses mentioned above were used as positive controls. In addition sera were tested for the presence of virus-neutralizing antibodies specific for A/PR/8/34 using a micro virus-neutralization (VN) assay using 96 wells plates (Greiner Bio-One) as described previously [605].

Lung virus titers

Lungs were snap frozen on dry ice with ethanol and stored at -80°C. Lungs were homogenized using a FastPrep-24® (MP Biomedicals, Eindhoven, The Netherlands) in transport medium (MEM with Hanks' balanced salt solution (Lonza, Basel, Switzerland) containing 0,5% lactalbumin enzymatic hydrolysate (Sigma-Aldrich, Zwijndrecht, The Netherlands), 200 U/ml penicillin (Lonza), 200 µg/ml streptomycin (Lonza), 250 µg/ml gentamycin (Life technologies, Bleiswijk, The Netherlands), 100 U/ml polymyxin B sulfate (Sigma-Aldrich), 50 U/ml nystatin (Sigma-Aldrich) and 10% glycerol (Sigma-Aldrich)) and centrifuged briefly. Quadruplicate ten-fold serial dilutions of the lung samples were used to determine the virus titers in a confluent layer of MDCK cells in 96 wells plates (Greiner Bio-One) as described previously [545].

Detection of virus-specific $CD8^+$ T cells by dextramer (Dm)-staining

Single-cell splenocyte suspensions were prepared as described previously [338]. Splenocytes were washed with 5% FBS in PBS and stained for flow cytometry with fluorchrome-labelled monoclonal antibodies (mAbs) specific for selected cell differentiation markers: CD3e-PerCP, CD8b.2-FITC (BD Pharmingen, Breda, The Netherlands) and H-2Db dextramers with the $NP_{366-374}$ epitope (ASNENMETM) (APC-labeled) or the $PA_{224-233}$ epitope (SSLENFRAYV) (PE-labeled) (Immudex, Copenhagen, Denmark) and LIVE/DEAD aqua Fixable Dead Cell stain (L/D) (Invitrogen, Breda, The Netherlands). After extensive washing cells were analyzed using a FACSCantoII flowcytometer and FACS Diva software (BD Biosciences, Breda, The Netherlands)

Peptides and intracellular interferon gamma (IFN-γ) staining

Splenocytes were cultured in the absence or presence of 5µM peptide $NP_{366-374}$ (ASNENMETM) or $PA_{224-233}$ (SSLENFRAYV) (immunograde >85% purity) (Eurogentec, Maastricht, The Netherlands) and were subsequently stained with CD3e-PerCP, CD8b.2-FITC, L/D and IFN-γ-PacificBlue (Biolegend, London, United Kingdom) as described previously [487]. Cells were analyzed using a FACSCantoII flowcytometer and FACS Diva software.

Human monocyte-derived DCs collection and in vitro maturation

Peripheral blood mononuclear cells (PBMCs) from three healthy blood donors (18 to 64 years of age) (Sanquin Bloodbank, Rotterdam, The Netherlands) were isolated and cultured for 6 days to obtain immature human DCs as described previously [649]. Subsequently these cells were stimulated o/n at 37°C with PBS (negative control), G3 [20µg/ml], G3/DT [20µg/ml] or LPS [1µg/ml] (positive control) (Sigma-Aldrich). After stimulation, cells were stained for CD80-FITC, CD83-APC and CD86-PE (BD Pharmingen) or CD11c-APC, HLA-DR-PerCP (BD Biosciences) and 2β-microglobulin-PE (BD Pharmingen) and expression was

determined by flow cytometry using a FACSCantoII flowcytometer and FlowJo software (FlowJo, Ashland, USA). Experiments were performed in quadruplicate. Culture supernatants from the above mentioned DC-stimulations were used to assess the concentrations of TNF-α, IFN-γ, IL12p70, IL1-β, IL-6, IL-4 and IL-10 via enzyme-linked immunosorbent assay (ELISA) using Ready-Set-Go ELISA-kits (eBioscience, Vienna, Austria).

Antigen presentation by human monocyte-derived DCs and BLCLs

Influenza virus-specific $CD8^+$ T cell clones directed against the HLA-A*0201 restricted $M1_{58-66}$ GILGFVFTL epitope and the HLA-B*2705 restricted $NP_{174-184}$ RRSGAAGAAVK epitope were generated as described previously [425].

Immature human DCs of an HLA-typed healthy blood donor were obtained as described above. HLA-typed immature human DCs or B lymphoblastoid cell lines (BLCLs) were incubated o/n at 37°C with vaccine [40µl/ml] only or in combination with G3 [20µg/ml] or G3/DT [20µg/ml]. Negative controls were incubated with PBS, G3 or G3/DT only and positive controls were incubated with 100µM peptide (GILGFVFTL or RRSGAAGAAVK) (Eurogentec) or infected with A/PR/8/34 (MOI 3). The DCs and BLCLs were used as target cells for the stimulation of influenza virus-specific $CD8^+$ T cell clones. IFN-γ responses of *in vitro* stimulated $CD8^+$ T cell clones were determined by ELISpot assay as well as intracellular staining (ICS) for IFN-γ.

The IFN-γ ELISpot assay was performed according to manufacturer's instructions (Mabtech Nacka Strand, Sweden) with an effector-to-target (E:T) ratio of 1:3 in duplicate as described previously [536].

For the ICS, cells of the respective $CD8^+$ T cell clones were stimulated with targets cells (E:T of 1:5) in duplicate for 6hr at 37°C in the presence of Golgistop. Cells were stored o/n at 4°C and subsequently stained with fluorchrome-labeled mAbs CD3-PerCP (BD Bioscience), CD8-FITC (Dako, Glostrup, Denkmark) and L/D in the presence of Golgistop, fixed and permeabilized using Cytofix and Cytoperm and stained with IFN-γ-PE (BD Pharmingen). Cells were analyzed with a FACSCantoII flowcytometer and FACS Diva software.

Statistical analysis

Data for weight loss, lung virus titers, dextramer staining and IFN-γ staining between pairs of groups were analyzed using the Mann-Whitney U test. Time to death were compared using the Kaplan-Meier curves and the log-rank test. Differences were considered significant at $p<0.05$.

Results

G3 and G3/DT enhance the vaccine-induced antibody response

Upon vaccination none of the mice displayed any signs of discomfort. Limited weight loss (<2%) was observed during two days post vaccination in some mice randomly distributed over the experimental groups, including those that received PBS only (data not shown).

All mice that received the trivalent inactivated influenza vaccine, with or without adjuvant developed HI antibody titers against the three vaccine strains (A/California/7/2009, A/Victoria/361/2011 and B/Hubei-Wujiang/158/2009) after the first vaccination (Figure 1A, B and C). However, for all vaccine strains the geometric mean titers (GMTs) of mice that received the vaccine with adjuvant G3/DT or G3 (group 1 and 2, respectively) were significantly higher than those of mice that received the unadjuvanted vaccine (group 3). Four weeks after the second vaccination the GMTs against the vaccine strains increased in the mice of group 3.

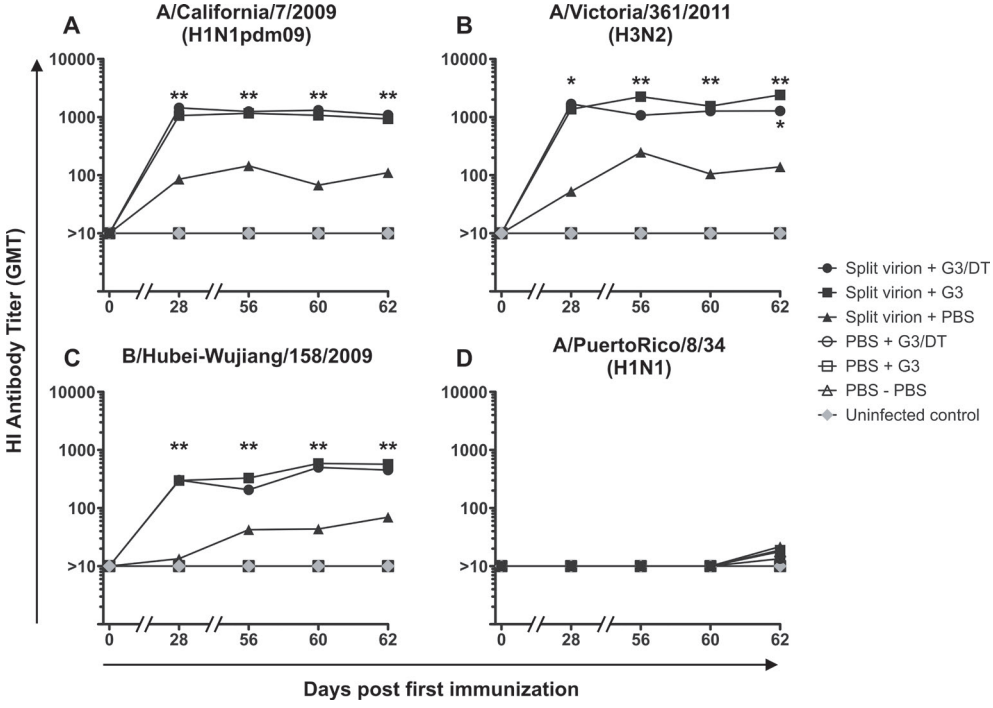

Figure 1 Geometric mean serum antibodies titers after immunization.

Serum antibody titers against vaccine stains A/California/7/2009 (A), A/Victoria/361/2011 (B) and B/Hubei-Wujiang/158/2009 (C) and the A/PR/8/34 challenge stain (D) were determined before immunization (T=0), 28 days after the first immunization and 28 days after the second immunization (day of challenge with A/PR/8/34) and day of euthanasia (4 and 6 days after challenge) by HI assay. (*) and (**) indicates that the difference in antibody titers in mice immunized with the split virion vaccine adjuvanted with G3 or G3/DT and split virion only was statistically significant (p<0.05 and p<0.01 respectively). The only significant difference between the split virion immunized mice with the G3 adjuvant versus the split virion immunized mice adjuvanted with the G3/DT adjuvant was observed in the A/Victoria/361/2011 strain at day 62 (*; p<0.05).

Although the GMTs of group 1 and 2 that received the G3/DT and the G3 adjuvanted vaccine respectively, did not increase after the second vaccination, they remained significantly higher than the GMTs of group 3 (no adjuvant) (p<0.01). No differences in GMTs were detected between group 1 and 2, except for antibody titers against A/Victoria/361/2011 on day 56, which were higher in mice of group 2 (p<0.05). No HI antibodies were detected in any of the control mice (groups 4, 5, 6 and 7). In none of the mice, vaccine-induced antibodies were detected that cross-reacted with the influenza virus strain A/PR/8/34 that was used for challenge infection of the mice (Figure 1D). This was confirmed with a VN assay (data not shown).

G3/DT improves the protective efficacy of the split virion vaccine against an antigenically distinct influenza virus

Four weeks after the second vaccination mice were inoculated with a lethal dose of influenza A/PR/8/34, except for control group 7. All groups displayed similar weight loss, starting at day 2 p.i. until day 4 p.i. (Figure 2A). From day 5 p.i. onwards, mice vaccinated with the G3/DT-adjuvanted vaccine (group 1) started to gain weight, while mice of the other groups continued to lose weight up to day 6 p.i. From day 5 onwards, the body weight of mice from group 1 remained significantly higher compared to that of mice from group 2 and 3 that received the G3-adjuvanted and unadjuvanted vaccine respectively, (p<0.01).
All mice from group 1 survived until day 6 p.i. while only 20% and 0% of the mice survived of group 2 and group 3 respectively (p<0.01) (Figure 2B).

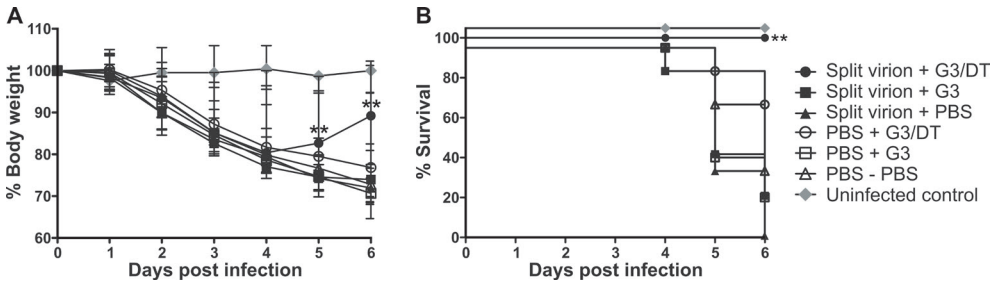

Figure 2 Outcome of inoculation with influenza virus A/PR/8/34
Mice immunized with split virion adjuvanted with G3/DT (group 1; black circles), with G3 (group 2; black squares) or unadjuvanted (group 3; black triangles) and their controls G3/DT only (group 4; open circles), G3 only (group 5; open squares) and PBS (group 6; open triangles) as well as unchallenged mice (group 7; grey diamonds) were weighted daily and mean weight loss was calculated (A). (**) indicates a significant increase in mean bodyweight in group 1 at day 5 and 6 post inoculation (p<0.01) compared to all other groups. Animals were euthanized according to pre-set humane endpoints and were recorded as dead to calculate the cumulative survival after A/PR/8/34 challenge (B). (**) indicates a significantly higher survival rate of the mice vaccinated with the G3/DT adjuvanted split virion vaccine (p<0.01).

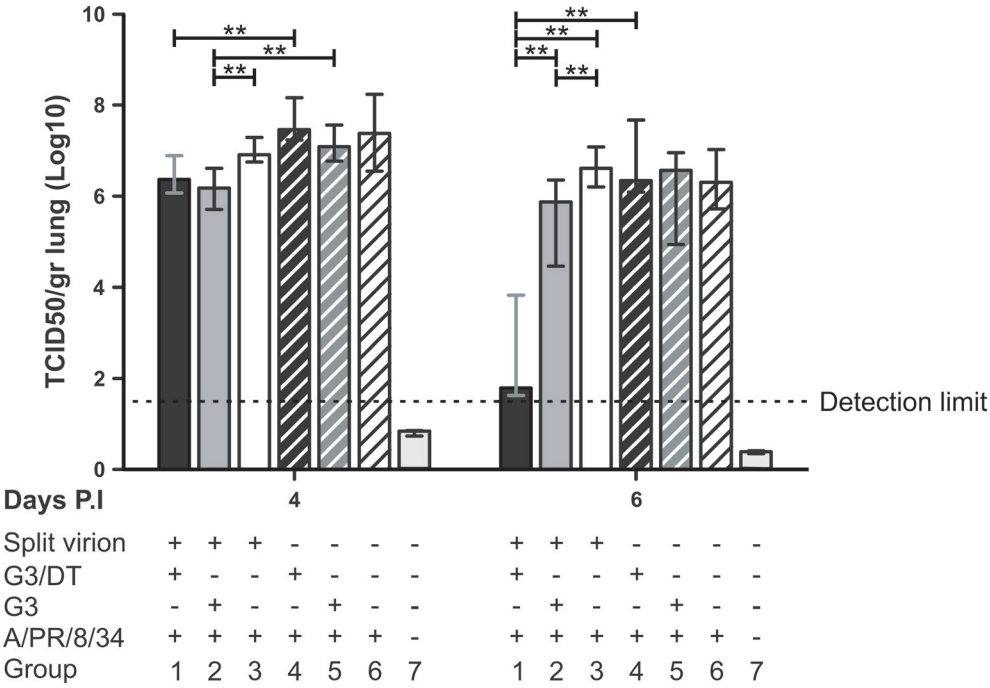

Figure 3 Virus titers in the lungs after A/PR/8/34 virus inoculation
Mean and range of lung virus titers of each group at day 4 and 6 post inoculation (p.i.) were determined. The different groups are indicated underneath the figure. Dotted line (- - -) indicates the detection limit. Statistically significant differences are indicated (**; $p<0.01$).

G3/DT adjuvanted vaccine-induced immunity restricts virus replication

Lung virus titers were assessed on day 4 and 6 p.i. (Figure 3). On day 4 p.i. mice of group 1 ($10^{6,4}$ $TCID_{50}$) and 2 ($10^{6,2}$ $TCID_{50}$) (G3/DT or G3-adjuvanted vaccine respectively) had significantly lower virus titers compared to their PBS control groups 4 ($10^{7,5}$ $TCID_{50}$; $p<0.01$) and 5 ($10^{7,1}$ $TCID_{50}$; $p<0.01$), while no statistically significant difference was detected between the unadjuvanted group 3 ($10^{6,9}$ $TCID_{50}$) and the PBS control group 6 ($10^{7,4}$ $TCID_{50}$). No statistically significant difference was detected between group 1 and 2. Group 2 (G3 adjuvanted vaccine) had a significantly lower mean titer than group 3 (unadjuvanted vaccine) ($p<0.01$). The most remarkable difference in lung virus titers was observed on day 6 p.i.: The mean virus titer of group 1 (G3/DT adjuvanted vaccine) was $10^{1,8}$ $TCID_{50}$, which was significantly lower than that of control group 4 ($10^{6,3}$ $TCID_{50}$), but also than that of group 2 ($10^{5,9}$ $TCID_{50}$) and 3 ($10^{6,6}$ $TCID_{50}$) ($p<0.01$). A smaller but significant reduction in lung virus titer was also observed between group 2 and group 3 ($p<0.01$).

Detection of virus-specific CD8⁺ T cells by Dm-staining in the G3/DT adjuvanted vaccine group only

Since HI antibodies against A/PR/8/34 were undetectable in all mice at day of inoculation (Figure 1D), we wished to investigate whether virus-specific CD8⁺ T cells had contributed to the protection observed in mice that received the G3/DT-adjuvanted vaccine. Dextramer-staining was used to measure the frequency of CD8⁺ T cells specific for the $NP_{366-374}$ and the $PA_{224-233}$ epitope on day 6 p.i. (Figure 4). Both epitopes are present in the A/PR/8/34 backbone of the influenza A vaccine strains and the A/PR/8/34 virus used for challenge infection. The mean frequencies of $NP_{366-374}$ and $PA_{224-233}$ specific CD8⁺ T cells were significantly higher in mice of group 1 that received the G3/DT adjuvanted vaccine (7.5% and 3.0%, respectively) than in mice of group 4 (G3/DT adjuvant only) (1.2% and 2.1%, respectively; $p<0.01$). These frequencies also exceeded those observed in mice of group 2 (G3 adjuvanted vaccine) ($NP_{366-374}$ 1.7% and $PA_{224-233}$ 2.2%; $p<0.05$) and those observed in group 3 (unadjuvanted vaccine) ($NP_{366-374}$ 1.4% and $PA_{224-233}$ 2.3%; $p<0.01$). A small but significant difference ($p<0.05$) was observed for $NP_{366-374}$ between group 3 and group 6 (no vaccination) (1.0%).

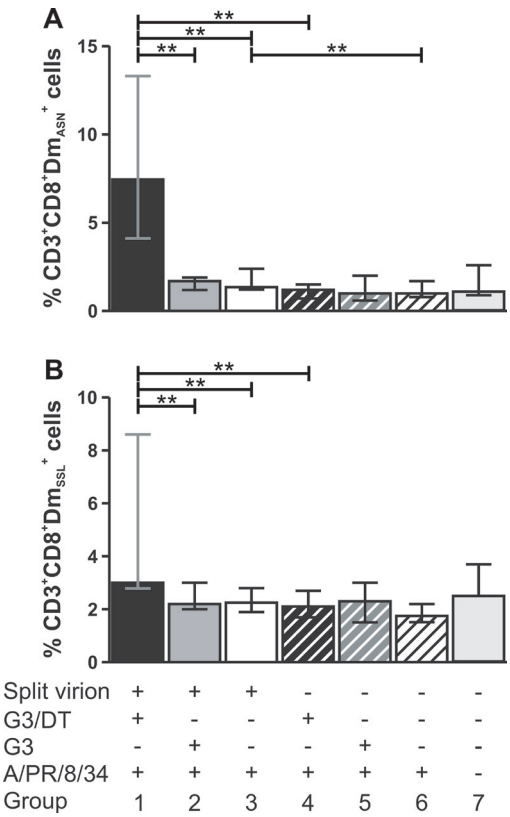

Figure 4 Dextramer staining of virus-specific CD8⁺ T cell splenocytes.
The mean and range of the proportion of Dm-NP_{ASM}^{+} cells (A) and Dm-PA_{SSL}^{+} cells (B) in the $CD3^{+}CD8^{+}$ T cell population was determined on day 6 p.i.. Statistically significant differences are indicated (**; $p<0.01$).

Detection of virus-specific CD8⁺ T cells by intracellular IFN-γ staining in the G3/DT adjuvanted vaccine group only

To determine the frequency of $NP_{366-371}$ and $PA_{224-233}$ specific CD8⁺ T cells, intracellular IFN-γ staining was performed. To this end, splenocytes obtained on day 6 p.i. were stimulated with synthetic peptides $NP_{366-371}$ and $PA_{224-233}$ and the percentage of IFN-γ positive cells was measured (Figure 5). The highest response was observed for the $NP_{366-371}$ peptide in group 1 (G3/DT adjuvanted vaccine) (22.2%; $p<0.01$). In none of the other groups a $NP_{366-371}$ peptide-specific CD8⁺ T cell response could be detected. In none of the groups a significant CD8⁺ T cells response to the $PA_{224-233}$ epitope was observed.

Both G3 and G3/DT have a similar effects on maturation of human monocyte-derived DCs

In vivo results clearly showed that both G3 and G3/DT promoted antibody responses after vaccination with inactivated split virion vaccine, but only G3/DT was able to induce CD8⁺ T cell responses. Since the mechanism underlying this discrepancy is unknown we wished to address this *in vitro*. Since ISCOM adjuvants are known to have an effect on maturation and the cytokine response of APCs [650, 651] we determined the effect of G3 and G3/DT on the maturation of immature

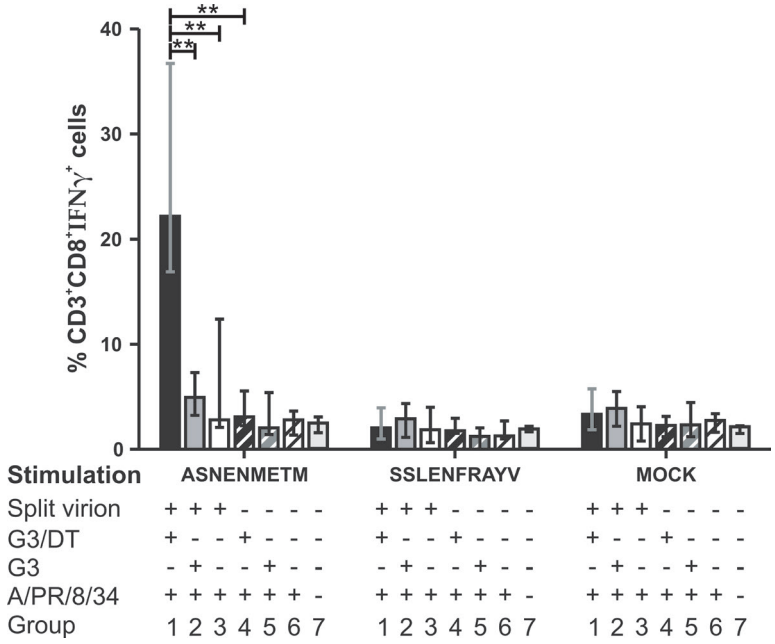

Figure 5 Intracellular IFN-γ staining of CD3⁺CD8⁺ T cell splenocytes

Mouse splenocytes were stimulated either with the ASNENMETM or SSLENFRAYV influenza peptides or mock stimulated as a negative control. Frequency of CD3⁺CD8⁺ splenocytes specific for influenza peptides $NP_{366-374}$ (ASNENMETM) and $PA_{224-233}$ (SSLENFRAYV) were determined by intracellular IFN-γ staining. Statistically significant differences between groups are indicated (**; $p<0.01$).

human DCs (Figure 6A). Both G3 and G3/DT enhanced the levels of CD83, a DC maturation marker, *in vitro*. Both adjuvants also equally enhanced the expression of HLA class I and II molecules on the DCs surface. Neither G3 or G3/DT affected the expression of T cell co-stimulatory markers CD80 and CD86 on the surface of human DCs. In addition cytokine production by DCs stimulated with G3 or G3/DT was similar (data not shown).

Both G3 and G3/DT are able to enhance antigen presentation

Next, we tested if G3/DT could improve antigen presentation and activation of $CD8^+$ T cells *in vitro*. To this end, *in vitro* immature HLA-typed human DCs and HLA-typed BLCLs were incubated with split virion vaccine with or without G3 or G3/DT and subsequently their ability to activate $CD8^+$ T cell clones specific for influenza $M1_{58\text{-}66}$ or $NP_{174\text{-}184}$ epitopes was determined. As shown by ELISpot (Figure 6B, C, D) and ICS (Figure 6E, F) G3 and G3/DT promoted the activation of the influenza virus-specific T cell clones in the presence of the split virion vaccine that in the absence of adjuvant failed to activate virus-specific $CD8^+$ T cells efficiently.

Discussion

In the present study, it was demonstrated that the G3/DT adjuvant improved the antibody response to a trivalent inactivated split virion vaccine in mice and promoted virus-specific $CD8^+$ T cell responses. Furthermore, G3/DT improved the protective efficacy of the vaccine against a lethal infection with the antigenically distinct influenza virus A/PR/8/34. Since the unadjuvanted vaccine and the vaccine adjuvanted with G3 only failed to induce protective immunity, the addition of DT was pivotal for the vaccine-induced protection. However, both the G3 and G3/DT adjuvant promoted the induction of vaccine-induced antibody responses. These antibodies did not cross-react with the challenge virus strain A/PR/8/34 in HI and VN assays, which explains the failure to induce sterile immunity and the inability to protect mice from weight loss after infection with A/PR/8/34. However, mice that received the G3/DT-adjuvanted vaccine started to gain weight and displayed 100% survival. The mean lung virus titer of mice that received the G3/DT-adjuvanted vaccine was significantly reduced on day 6 p.i. compared to the other groups. This accelerated clearance of infection in the G3/DT-adjuvanted vaccine group might be attributed to the presence of cross-reactive T cell immunity. Indeed the kinetics of virus replication resembled that of mice in which cross-protective T cell responses were induced by primary influenza virus infection [338, 339]. The induction of influenza A virus-specific $CD8^+$ T lymphocytes in mice that received the G3/DT-adjuvanted vaccine was confirmed and was not detectable in mice of any of the other groups. The mechanism underlying the induction of virus-specific $CD8^+$ T cell responses by the addition of G3/DT remains unclear. Human instead of mouse DCs were used to unravel the underlying mechanism because the use of human DCs

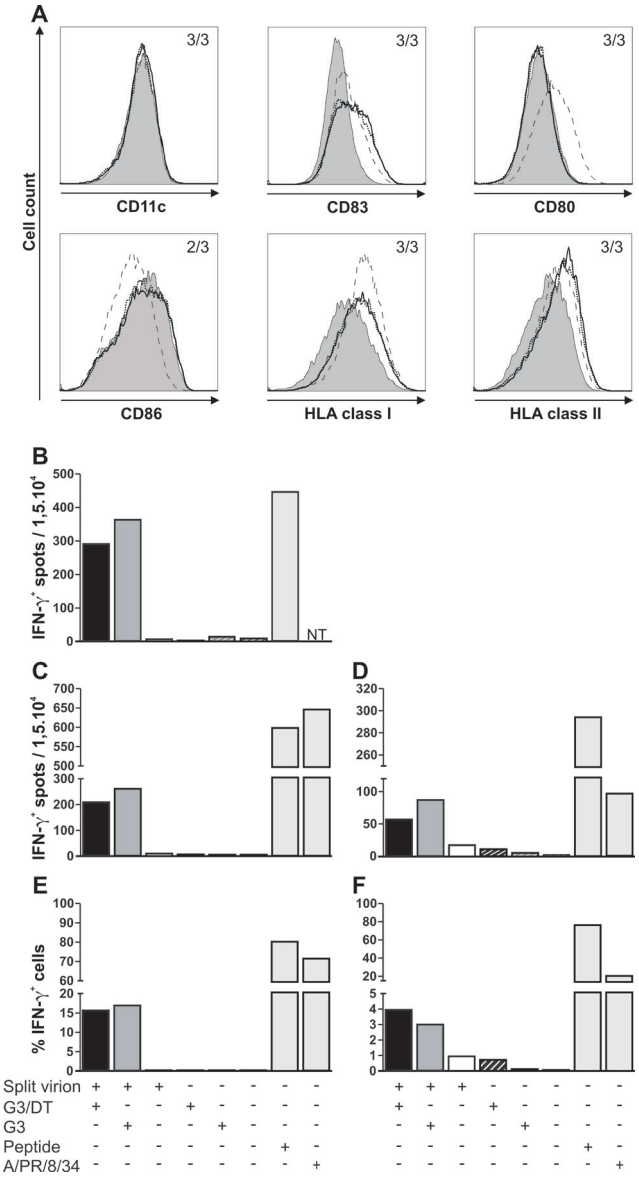

Figure 6 Effect of G3 and G3/DT on the maturation of human DCs and antigen presentation

A) Maturation of human HLA-un-typed DCs. DCs were unstimulated (grey curve) or treated with G3 (—), G3/DT (···) and LPS (---) and the surface expression of CD11c, CD83, CD80, CD86, HLA class I and II molecules was monitored. Results representative for three human subjects are shown. Numbers in the upper right corner of each histogram indicate how many of the three subjects display this particular result. In case of CD86 two out of the three subjects showed no difference in CD86 expression when DCs were stimulated with G3 or G3/DT whereas in one subject CD86 increased after G3 and G3/DT stimulation. Experiments were performed in quadruplicate.

Antigen presentation by human HLA-typed DCs (B, C, E) and BLCLs (D, F) after stimulation with split virion vaccine adjuvanted with G3, G3/DT or without adjuvant and their ability to stimulate $CD8^+$ T cell clones directed against the HLA-A*0201 restricted $M1_{58-66}$ GILGFVFTL epitope (B) or the HLA-B*2705 restricted $NP_{174-184}$ RRSGAAGAAVK epitope (C, D, E, F) was determined in an IFN-γ ELISpot assay (B, C, D) and by ICS of IFN-γ (E, F). Experiments were performed in duplicate.

not only allowed studying DC activation, but also antigen processing and presentation using influenza virus-specific T cell clones. Furthermore, it was shown previously that CD80 and CD86 expression in human and mouse DCs upon stimulation with an adjuvant, resembled each other [649]. Both G3 and G3/DT promoted the activation of virus-specific T cells *in vitro*. Most likely, both adjuvant preparations are able to introduce viral proteins into the cytosol of APCs, induce maturation of DCs and up-regulate MHC class I and II expression. The addition of DT in combination with the vaccine dose used *in vivo* may have tipped the balance in favor of inducing influenza virus-specific T cell immunity, which may have not been reflected properly in the *in vitro* experiments.

Although inactivated trivalent influenza vaccines are safe and able to elicit sufficient virus strain-specific neutralizing antibody responses, they fail to induce broadly protective T lymphocyte responses efficiently [476, 495, 640, 652, 653]. Their use even may prevent induction of cross-protective T cell immunity in naïve subjects otherwise induced by natural influenza virus infections [485]. Therefore, currently used inactivated influenza vaccines could benefit from the use of adjuvants, like G3/DT, in order to induce more broadly protective immune responses.

It should be realized that the influenza A virus vaccine strains share their back-bone genes with those of the challenge virus A/PR/8/34, which contributes to cross-reactivity of the T cell response and a favorable outcome of the challenge infection. However, T cell responses to the epitopes $NP_{366-371}$ and $PA_{224-233}$ also contribute to heterosubtypic immunity as was shown previously [337, 339]. Furthermore, it is likely that CTL responses against other conserved epitopes were elicited after vaccination with the G3/DT-adjuvanted vaccine, which are likely to have contributed to protective immunity.
Further research is needed to establish the exact mechanism that enables G3/DT but not G3 to induce virus-specific T cell responses *in vivo* and to confirm that G3/DT-adjuvanted split virion influenza vaccines also elicit cross-reactive T cell responses in humans.
Since mice that received the G3-adjuvanted vaccine without the DT component were not protected, although these mice developed similar strong antibody responses as mice in the G3/DT vaccine group, it is unlikely that antibodies to HA, NA or any other viral proteins contributed to the protection observed in the latter group. Although it has been shown that antibodies to A/H1N1 viruses that circulated before 1957 can cross-react with H1N1pdm09 viruses and vice versa [599, 654, 655], we were unable to detect vaccine (containing the H1N1pdm09 component)-induced antibodies that cross-reacted with the A/PR/8/34 challenge virus by HI or VN assays. Both the G3 and G3/DT adjuvant greatly enhanced the antibody response to all vaccine strains after a single immunization. Since a subsequent

immunization did not have a pronounced booster effect, a single dose of the adjuvanted vaccine may be sufficient to induce protective antibody responses, provided that the vaccine is matching the strain causing the infection antigenically. This is especially relevant in case of an emerging pandemic, when the timely availability of sufficient vaccine doses is desired [482, 484, 656-658]. Furthermore, it has been demonstrated that more than one immunization with possibly a high dose of a pandemic vaccine is required for the induction of protective immunity [484, 657, 658]. The G3 and G3/DT adjuvant may allow substantial antigen dose sparing. In the present study a dose of 5µg HA was sufficient to induce potent antibody responses.

In conclusion, G3/DT is a promising adjuvant formulation that not only potentiates the antibody response induced by influenza vaccines, but that also induces T cell immunity which could afford broader protection against antigenically distinct influenza viruses. These properties are not only desirable for protection against matching and antigenically mismatched seasonal influenza viruses, they may also contribute to protective immunity against influenza A viruses of alternative subtypes, which continue to pose a pandemic threat [88, 125, 147, 148]. Further (clinical) evaluation of G3 and G3/DT-adjuvanted influenza vaccines seems therefore warranted.

Acknowledgements

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CHAPTER 7:

Summarizing Discussion

Influenza virus infections are an important cause of respiratory tract disease in humans [2]. Seasonal influenza viruses cause an estimated 3-5 million severe clinical infections and result in 250,000-500,000 fatal cases annually [65, 66]. In general, the majority of these severe cases are caused by A/H3N2 viruses followed by influenza B and A/H1N1 virus infections [72-78]. Continuous antigenic drift enables these viruses to escape from recognition by virus-specific antibodies elicited after natural influenza virus infection or vaccination [67-70]. These antibodies also provide little to no protection in case of a pandemic outbreak with an antigenically distinct influenza virus of a novel subtype, like A/H5N1 or A/H7N9 [116, 139, 659]. This underscores the importance of increasing our knowledge of other correlates of protection that contribute to broadly protective immunity.
This thesis focuses on the role of $CD8^+$ cytotoxic T lymphocytes (CTLs) in the cross-reactive immune response. In chapter 2 we determined the cross-reactivity of influenza B virus-specific $CD8^+$ T cells with intra-lineage drift variants and with viruses of the opposing lineage. The cross-reactivity of virus-specific $CD8^+$ T cells, induced after infection with seasonal influenza A viruses, with the newly emerging A/H7N9 virus was assessed in chapter 3. In addition, the longevity of these cross-reactive CTL responses was determined (Chapter 4). In chapter 5 extra-epitopic amino acid substitutions were defined which play a role in evading recognition of the highly conserved $M1_{58\text{-}66}$ $CD8^+$ T cell epitope. Finally, in chapter 6, the potential of a novel adjuvant, G3/DT, to promote $CD8^+$ T cell responses after vaccination with a seasonal split virion vaccine was tested.

Heterosubtypic $CD8^+$ T cell responses in humans

In the absence of pre-existing antibody immunity, in the case of antigenic drift or shift, $CD8^+$ T cells form an important second line of protection against severe illness and even mortality as they are essential for viral clearance [344-347, 612, 613]. It is well known that influenza virus-specific $CD8^+$ T cells are mainly directed against epitopes of more conserved internal proteins of influenza viruses, like the NP and M1 protein [311, 314, 315, 344, 534, 612, 660]. The protective role of $CD8^+$ T cells in influenza virus infections has been extensively studied in mice and other animal models, which demonstrated that $CD8^+$ T cells contribute to homo- and heterosubtypic immunity [335-341, 538-540, 661, 662]. Human influenza A virus-specific $CD8^+$ T cells, induced after seasonal influenza A virus infections, have been shown to cross-react with swine origin triple-reassortant influenza A H3N2 virus (vH3N2), the 2009 pandemic H1N1 virus (H1N1pdm09) and the avian H5N1, H5N2 and H1N1 viruses *in vitro* [316, 317, 535, 541, 660]. These studies focused on the cross-reactive potential of seasonally induced $CD8^+$ T cells between antigenically distinct influenza A virus strains while the role of CTLs in influenza B virus infections remained largely unknown. In chapter 2 of this thesis, it was demonstrated that human influenza B virus-specific $CD8^+$ T cells, induced by

previous infections with seasonal influenza B viruses, displayed a high degree of cross-reactivity with intra-lineage drift variants and influenza B viruses belonging to the opposing lineage. The specificity of these cross-reactive CTLs remains largely unknown. Only a handful of NP derived, HLA-A*0201 and HLA-B*0801 restricted, influenza B virus $CD8^+$ T cell epitopes have been identified based on epitope prediction studies [542-544, 559]. Although we established that all these epitopes are highly conserved between both influenza B lineages, we could not confirm the functionality of the previously described HLA-B*0801 restricted epitopes in our study subjects. Nevertheless, it is tempting to speculate that cross-reactive $CD8^+$ T cells, induced by seasonal influenza B virus infections, may afford some degree of protection against a subsequent infection with an antigenically distinct influenza B virus. Thus far, no data is available to confirm that cross-reactive influenza B virus-specific CTLs reduce severity of disease caused by influenza B virus of the opposing lineage.

In chapter 3 we demonstrated that seasonal H3N2 (sH3N2), sH1N1 and H1N1pdm09 influenza A virus-specific human $CD8^+$ T cells display considerable cross-reactivity with the newly emerging avian A/H7N9 virus that causes frequent outbreaks among humans in China since 2013. Thus, humans who were previously infected with seasonal influenza A viruses possess influenza virus-specific $CD8^+$ T cells that cross-react with the heterosubtypic A/H7N9 virus and may afford a certain degree of protection against severe disease in case this A/H7N9 virus would become pandemic. Fortunately, most individuals will have encountered at least one seasonal influenza A virus infection by the age of six [287]. However, a recent study demonstrated that the strength of a cross-reactive CTL response against the novel A/H7N9 virus also depends on the ability to present conserved influenza A virus epitopes, which is determined by the HLA class I make-up of an individual and varies between ethnicities. Based on these results it was predicted that an average of 37% of the human population should have pre-existing CTLs that can cross-react with the A/H7N9 virus with the highest prevalence in the Caucasian population (57%) [537]. Also, all of the healthy study subjects investigated in chapter 3 possess at least 2-3 of these preferred HLA class I alleles, which explains their robust CTL response to the A/H7N9 virus. The protective nature of the cross-reactive influenza A virus-specific CTLs, induced after a previous infection with an antigenically distinct influenza A virus, was demonstrated in mice where these cells protected them from a lethal A/H7N9 challenge [662, 663]. An early CTL response was recently correlated to a more rapid recovery of A/H7N9 virus infected patients [613]. Although it is tempting to speculate that these patients benefited from a rapid recall response of heterosubtypic CTLs, the presence and frequency of these cross-reactive CTLs before the patients were infected with the A/H7N9 virus was not established, an increase in $CD8^+$ T cells as a result of proliferation of previously naïve CTLs could therefore not be excluded. Also, a faster recall CTL response was

observed in patients infected with the H1N1pdm09 virus [344, 603]. Furthermore, current laboratory-confirmed A/H7N9 cases are greatly skewed towards severe cases in predominant elderly patients, therefore it is likely that we currently overlook the true protective effect of the heterosubtypic CTL response, as mild infections might not be reported. Recent studies have demonstrated that 6.3% to 14.9% of the poultry workers living in A/H7N9 endemic areas are seropositive for antibodies against the emerging A/H7N9 virus, suggesting that subclinical infections do occur [664, 665]. It is tempting to speculate that these asymptomatic poultry workers might have benefited from a robust heterosubtypic recall CTL response that protected them from sever disease.

Importance of a long-lived human influenza A virus-specific CD8$^+$ T cell response in preventing severe disease during a pandemic outbreak

Currently, little is known about the longevity of these cross-reactive influenza A virus-specific CTL populations in humans. Although the longevity of the CTL response in humans was never properly established, it was previously suggested that these virus-specific memory CTL responses might wane over time, based on the observation that the T cell population with lytic capacity rapidly declined after an influenza virus infection [666]. However, this is in contrast with studies in mice which demonstrated that influenza A virus-specific T cell memory can be maintained for life [604]. The decline of the lytic CTL population in humans could also be attributed to the reduced lytic capacity exerted by memory CTLs. In chapter 4, we investigated the longevity of the human influenza A virus-specific CD8$^+$ T cell populations, using unique PBMC samples obtained from HLA-typed healthy study subjects collected between 1999 and 2012. We demonstrated that human influenza A virus-specific CTL responses are long-lived and that intercurrent influenza A virus infections temporally increase in the frequency of influenza A virus-specific effector T cells, effector memory T cells (T_{EM}) and effector memory RA T cells (T_{EMRA}). The proportion of these subsets decreases in the contraction phase after which small numbers of T_{EM}, T_{EMRA} and central memory T cells (T_{CM}) persist in the following years. Although the proportion of influenza A virus-specific CD8$^+$ T cells was small, it is comparable to the proportion of memory CD8$^+$ T cells detected after other acute viral infections [343, 606-608, 667]. Also, it is likely that we have underestimated the real number of persisting memory CD8$^+$ T cells, since these cells preferentially reside in the lymph nodes and/or in the lung rather than in peripheral blood [332, 334, 668-671]. Lung resident memory CD8$^+$ T cells resemble an effector-like phenotype in order to rapidly respond to a secondary infection, while lymph node resident memory CD8$^+$ T cells, mostly of T_{CM} phenotype, rapidly expand for a sufficient recall response [672-674]. Yet, this does not answer the question whether the low frequencies of influenza A virus-specific CTLs found in the peripheral blood of our healthy study subjects can be correlated to the prevention of severe disease in case of an infection with

an antigenically distinct influenza A virus. It was previously suggested that a LAIV induced arbitrary threshold level of ≥100 SFU/10^6 PBMC is required for effective T cell mediated protection against clinical influenza virus infection in children [675]. Furthermore, the 2009 pandemic H1N1 outbreak enabled an unique opportunity to study the role of human pre-existing CTLs in heterosubtypic immunity against the antigenically novel H1N1pdm09 virus. Two independent studies demonstrated that a low baseline of pre-existing CTL populations in the peripheral blood, similar to the low baseline found in our study subjects, correlated with reduced viral shedding and a lower symptom score after infection with the H1N1pdm09 virus [344, 612]. Together these studies indicate that low levels of pre-existing influenza A virus specific CTLs in the peripheral blood are long-lived and could be predictive for the severity of an antigenically distinct influenza A virus infection.

Novel escape mechanism to evade recognition of a highly conserved $CD8^+$ T cell epitope

The high mutation rate of influenza viruses and the selective immune pressure exerted by virus-specific CTLs drive the accumulation of amino acid substitutions inside epitopes that are associated with evasion from recognition by these epitope-specific CTLs. Indeed, significantly more non-synonymous mutations are observed in CTL epitopes than in the rest of the viral nucleoprotein (NP), indicating that these epitopes are subjected to positive selection [410, 676]. Various studies have identified amino acid substitutions in T cell receptor (TCR) contact residues [422, 423, 426, 427, 441, 442] or at anchor residues [421-425, 440] that resulted in loss of recognition by epitope-specific CTLs. However, some influenza A virus CTL epitopes are highly conserved. One of these highly conserved epitopes is the HLA-A*0201 / HLA-C*0801 restricted matrix protein 1 $(M1)_{58\text{-}66}$ (GILGFVFTL) epitope [445-447]. Mutations to escape the $M1_{58\text{-}66}$-specific CTL response are to be expected, considering the high selective pressure exerted on the $M1_{58\text{-}66}$ epitope as a result of its immunodominant nature when presented by the HLA-A*0201 molecule [448], which has a prevalence of >40% in the Caucasian population [617]. Although the M1 protein of seasonal A/H3N2 viruses has circulated in the human population for almost 100 years (Chapter 5 Figure 1) [81-83, 86, 91, 101, 453, 454], viruses were unable to acquire mutations at TCR contact or anchor residues as these mutations were not tolerated without loss of viral fitness [410, 450].

A recent study demonstrated that the $M1_{58\text{-}66}$ epitope was abundantly expressed on the surface of virus infected HLA-A*0201 positive cells, which corresponds with the immunodominant nature of this epitope [625]. However, Keskin *et al.* suggest that the high expression of the $M1_{58\text{-}66}$ epitope in combination with the low functional avidity exerted by their $M1_{58\text{-}66}$-specific T cell line represents a decoy mechanism by which influenza A viruses try to prevent recognition of epitopes with a higher T cell avidity. They conclude that the conservation of this particular epitope is not the

result of functional constraints but merely represents an immune-evasion strategy by which influenza A viruses prevent the generation of more potent CTLs against other HLA-A*0201 restricted epitopes. However, the authors did not take in account that the $M1_{58-66}$ epitope largely overlaps with a highly conserved and functionally important nuclear export signal of the M1 protein, which could well explain why no CTL-escape mutations are found in nature [451, 452]. Furthermore, previous studies have demonstrated that $M1_{58-66}$-specific CTLs have the highest functional avidity of all conserved epitopes tested [448]. The low functional avidity observed by Keskin *et al.* might have been a result of how the $M1_{58-66}$-specific T cell line was obtained after stimulation with peptides, which may have selected for $M1_{58-66}$-specific T cells of low functional avidity [677]. Also, the authors fail to demonstrate that CTLs directed to other influenza HLA-A*0201 restricted epitopes have a higher functional avidity, or that these epitopes are more abundantly expressed on the influenza A virus infected cell surface in the absence of the $M1_{58-66}$ epitope. Furthermore, in contrast to what the authors claim, the overall CTL response is smaller in HLA-A*0201$^-$ subjects as compared to HLA-A*0201$^+$ subjects, as was demonstrated in groups of blood donors with matched HLA class I alleles [313].

Since several studies have clearly demonstrated that the $M1_{58-66}$ peptide represents a functional epitope, in which conventional CTL escape mutations in the anchor or TCR contact residues are not tolerated due to functional constrains, we hypothesize that influenza A viruses might utilize other escape mechanism(s) to evade CTL recognition of conserved epitopes like this one. In chapter 5, we demonstrated that the $M1_{58-66}$ epitope was less efficiently recognized by $M1_{58-66}$-specific CTLs when the M1 protein was derived from a human influenza A virus than when the M1 protein was derived from an avian influenza A virus. This indicates that human influenza A viruses have developed a strategy to evade the human $M1_{58-66}$-specific CTL response. Naturally occurring variations at extra-epitopic amino acid residues in a human influenza A virus were shown to delay and impair the activation and reduce lytic activity of the $M1_{58-66}$-specific CD8$^+$ T cells, while retaining the conserved amino acid sequence of the epitope. Reciprocal exchange of the extra-epitopic amino acid residues between avian and human influenza A viruses partially reversed the CTL recognition pattern. We hypothesize that the difference in extra-epitopic amino acid residues either change the cleavage pattern of the M1 protein and/or define which protease processes the M1 protein, which will eventually determine the extent of $M1_{58-66}$ epitope presentation. This additional immune evasion strategy may help the influenza A virus to perpetuate in the human population in the presence of pre-existing virus-specific CTL immunity.

Lack of CTL evasion may have contributed to lower severity of the 2009 pandemic

Compared to the pandemics of 1918, 1957 and 1968, the pandemic of 2009 was generally considered milder [80, 89, 94, 102, 678]. Especially in the elderly, normally

at high risk for severe influenza A virus infections, the morbidity and mortality was relatively low [102]. The lower susceptibility of the elderly population to the 2009 pandemic influenza virus was attributed to cross-protective antibodies that they had acquired during a previous infection with an antigenically related A/H1N1 virus that circulated prior to 1957 [103, 240, 241]. Independent studies demonstrated that a higher prevalence of pre-existing cross-reactive CTLs directed against the 2009 pandemic influenza virus was inversely correlated with disease severity [344, 612, 660]. These studies did not take into account potential differential recognition of pandemic influenza viruses [345-347]. However, the extra-epitopic amino acid residues that were associated with evasion from the pre-existing $M1_{58-66}$-specific CTL response were absent in the M1 protein of the 2009 pandemic influenza virus (Chapter 5). This was in contrast to the influenza viruses that caused the more severe pandemics of 1918, 1957 and 1968, as these viruses contained these extra-epitopic amino acid residues. Consequently, the data presented in chapter 5 suggests that in previously infected HLA-A*0201$^+$ individuals, the 2009 pandemic viruses were better recognized by pre-existing $M1_{58-66}$-specific $CD8^+$ T cells, than the viruses that caused the pandemics of 1918, 1957 and 1968. Additionally, the high prevalence of the HLA-A*0201 allele in the human population may have further limited the spread of the virus in the human population.

Based on the findings in chapter 5 it is tempting to postulate that studies like these will help to predict the severity of the next major influenza pandemic, as screening for the presence or absence epitopic and extra-epitopic mutations that may hamper the presentation of these CTL epitopes potentially correlates with the strength of the pre-existing CTL response in the population.

Development of a broadly-protective influenza vaccine

The use of vaccines that elicit heterosubtypic immunity can offer some protection in case of seasonal antigenic drift or an emerging influenza pandemic. Current seasonal influenza vaccines contain two influenza A virus strains (A/H3N2 and A/H1N1) and one or two influenza B virus strains (B/Yamagata and/or B/Victoria lineage) [456]. The effectiveness of these vaccines relies on the induction of strain-specific antibodies that match the epidemic strains [476, 477]. However, continuous antigenic drift of seasonal influenza viruses prevents current vaccines to afford life-long protection [67-70] and necessitates an almost annual update of the influenza vaccine strains. Recommendations for vaccine strains of the upcoming influenza season are made months in advance due to the lengthy process of vaccine production [478]. Unforeseen antigenic drift among circulating influenza A and B viruses may affect vaccine effectiveness [479-481], as was recently the case for the A/H3N2 component of the 2014/2015 influenza vaccine used in the Northern hemisphere [679-681]. Furthermore, antibodies elicited after natural seasonal influenza virus infection or seasonal vaccination provide little to no protection in

case of the emergence of an antigenically distinct influenza virus of a novel subtype, like A/H5N1 or A/H7N9 [79]. This underscores the importance of inducing broad-protective heterosubtypic immune response after vaccination. An ideal influenza vaccine elicits homo- and heterosubtypic immune responses after a single administration with a low antigen dose and is safe to use in all risk groups.

Findings in this thesis highlight the importance of cross-reactive $CD8^+$ T cell responses in preventing severe disease in case of antigenic mismatch (**Chapter 2**) or against a potentially pandemic virus (**Chapter 3**). Vaccines that induce cross-reactive $CD8^+$ T cell-mediated immunity may offer another layer of long-lasting protection (**Chapter 4**) which is less sensitive to antigenic drift or shift. It has been demonstrated that live attenuated influenza vaccines can induce virus-specific $CD8^+$ T cells to some extent, in contrast to the more frequently used whole inactivated, split virion or subunit influenza vaccines [343, 476, 495, 560, 640, 652, 653]. Mouse models have indeed demonstrated that multiple doses of live attenuated influenza vaccines, and not inactivated or subunit influenza vaccines, can induce long-lived broad-protective immune responses [682-685]. It was recently confirmed in humans that humoral and cellular immune responses induced by a live attenuated influenza vaccine can be maintained for at least one year after immunization [686]. Longevity of these responses is likely to depend on the timing and route of immunization [671, 686-688]. The ability of live attenuated influenza vaccines to induce cross-reactive $CD8^+$ T cells in humans seems to be mostly limited to young children, possibly due to their naïve infection status [495, 560]. This indicates that vaccine immunogenicity could be improved. Internal proteins (backbone) of current live attenuated vaccines are derived from a cold adapted human influenza strain, namely A/Ann Arbor/6/60 [689, 690]. However, proteins derived from human influenza viruses are likely to possess mutations that hamper the presentation of CTL epitopes. This is in contrast to proteins derived from avian influenza viruses as they did not experience the necessity to evade the human CTL response (**Chapter 5**). Indeed, the A/Ann Arbor/6/60 influenza strain encompasses all human amino acid substitutions that were associated with evasion from the $M1_{58\text{-}66}$-specific CTL response, with exception of the amino acid residue at position 15. Whether the use of an avian backbone enhances the elicited broad-protective CTL response in humans remains to be established.

Although live attenuated vaccines are potentially more efficient in eliciting broad-reactive CTL responses, their administration is restricted in certain high-risk groups [464, 500]. Therefore, it would be of interest to develop inactivated influenza vaccine preparations that are able to induce CTL responses.

In **chapter 6** we tested whether a novel adjuvant (G3/DT) had the capacity to enhance the immunogenicity of a commercially available seasonal trivalent inactivated split virion influenza vaccine. It was demonstrated that G3/DT is a versatile adjuvant that significantly improved vaccine immunogenicity by

enhancing its antibody response and promoting the induction of virus-specific $CD8^+$ T cells. Mice that received the G3/DT-adjuvanted vaccine started to gain weight and displayed 100% survival after a lethal challenge with an antigenically distinct influenza virus. Also, their mean lung virus titers were significantly reduced by day 6 post infection. This was in contrast to mice that received an unadjuvanted vaccine. Since it was demonstrated *in vitro* that the elicited antibody response did not cross-react with the influenza virus used for the challenge, it was concluded that the protective effect was attributed to the induced influenza A virus-specific CTL response. These results demonstrate that the use of the G3/DT adjuvant in combination with inactivated vaccine formulations, like split virion and whole inactivated vaccines, would broaden the elicited immune response by inducing cross-reactive CTL responses. In addition, the G3/DT adjuvant may allow substantial antigen dose sparing as it strongly enhanced the elicited antibody response after a single vaccine dose. This is of special interest in case of an emerging pandemic, when the initial availability of sufficient vaccine doses might be limited, especially when the vaccines immunogenicity is low and would otherwise require multiple high dose immunizations in order to induce a protective immune response [482, 484, 656-658]. However, before these new vaccine strategies can be used in humans, they first need to be tested in clinical trials.

$CD8^+$ T cells have an important role in the protection against severe disease, especially in case of a pandemic outbreak, but are unable to afford sterile immunity [344-347, 612, 613]. Broader-reactive antibodies, directed against the stem region of the HA protein, have been identified for influenza A and B viruses [243-245, 248, 250-253, 691, 692] and current efforts are being made to develop universal influenza vaccines able to elicit these antibodies [246, 247, 249, 518, 519, 693-695]. Combining the induction of broad-reactive antibodies with the ability to induce an effective heterosubtypic CTL response may eventually result in the ultimate broadly-protective influenza vaccine which generates long-lasting protection against both seasonal and potentially pandemic influenza viruses.

Concluding remarks

The work described in this thesis provides new insights in the cross-protective immunity exerted by influenza A and B virus-specific CTLs and the results of these studies were discussed in the light of potential antigenic mismatches of seasonal influenza vaccines and current pandemic threats. Increased knowledge about the protective role of influenza virus-specific CTLs aids the development of universal influenza vaccines that in addition to broad-protective antibodies also elicit robust CTL responses. Furthermore, a better understanding of how CTLs contribute to heterosubtypic immunity may enable a more accurate prediction of the severity of the next emerging pandemic virus and possibly indicate which ethnicities will be most at risk of developing severe disease based on their HLA-profile and thus their

ability to present conserved CTL epitopes. A question that remains unanswered is why cross-reactive CTLs, seem to have offered so little protection during the devastating influenza pandemic of 1918? Especially since historical documentation and serological studies indicated that influenza virus infections resulted in frequent epidemics and pandemics prior to 1918, with the "Russian" influenza pandemic just 30 years earlier (1889-1892) [696-700]. Therefore, a large proportion of the 1918 population, with the possible exception of isolated countries/communities, would have encountered a previous influenza virus infection that resulted in the development of pre-existing cellular immunity. Furthermore, the presence of conserved CTL epitopes in the viral protein sequences of the 1918 influenza virus [537] and the ability of H1N1pdm09 influenza virus $NP_{418-426}$-specific CTLs to cross-react with the 1918 influenza virus [442] suggests pre-existing CTLs might indeed have had a potentially protective effect against severe infection with the 1918 influenza virus.

The suggestion that the pre-existing influenza virus-specific $CD8^+$ T cells are often short-lived and were therefore unavailable at the moment the 1918 influenza virus emerged [701], can be dismissed as we were able to demonstrate the longevity of influenza virus-specific $CD8^+$ T cells in healthy individuals (Chapter 4).

On the other hand, the strength of the cross-reactive CTL response may have varied between ethnicities, as genetic variations in HLA class I make-up influences the ability to present conserved influenza virus epitopes [537]. This would make some ethnic populations, like the Alaskan Natives and the Australian Aboriginals, more vulnerable for severe influenza virus infections, which is consistent with a high morbidity- and mortality-rates observed in these populations during the pandemic of 1918 [702] and 2009 [703, 704], although a combination of factors were likely to have influenced the disease severity in these populations [703, 704].

Others suggested that the pre-existing influenza virus-specific CTL response by itself was not able to cope with the extreme virulence of the 1918 pandemic virus and the rapid appearance of clinical disease [702].

An additional hypothesis that was not previously mentioned is possible immune suppression as a result of a recent measles virus infection [705-708]. Measles epidemics were frequently reported at the end of the 19^{th} and in the early 20^{th} century [709-713], including a large measles outbreak in the US military camps in the winter of 1917-1918 [709, 714]. The elderly population would have experienced measles in their childhood and pre-existing immunity would have protected them from contracting a measles virus infection in the years prior to the 1918 influenza pandemic. However, children and young adults who had not been previously infected would have been immunologically susceptible to a measles virus infection in the years preceding the 1918 influenza pandemic. Recent studies have demonstrated that the measles virus infects memory T lymphocytes, resulting in apoptosis and a prolonged state of immune suppression up to three years after

the initial measles virus infection [705-708]. Influenza virus-specific CTL responses were likely suppressed in young individuals who had endured a measles virus infection in the years prior to the 1918 influenza pandemic, making them more susceptible to a severe influenza virus infection. The combination of recovering from immunosuppression and an infection with an unexpected highly virulent virus might have contributed to severe inflammatory related pathology in a mechanism better known as the immune reconstitution inflammatory syndrome (IRIS) [696, 713, 715, 716]. Whether recent measles virus infections indeed lead to immunosuppression of the influenza virus-specific T lymphocyte response, resulting in a higher susceptibility to severe influenza virus infections and potential IRIS remains to be established. However, if this proved to be the case, people who have endured a recent measles virus infection would be at high risk of developing a severe influenza virus infection in case of an emerging pandemic. Fortunately, measles vaccines are widely available and have greatly reduced the prevalence of measles worldwide[708, 717, 718].

Collectively, the results obtained in the present studies confirm the cross-protective potential of $CD8^+$ T cells against developing a severe influenza virus infection. These results will aid the development of novel vaccine strategies and help policy makers to assess which individuals are most at risk for developing severe complications and therefore will be most in need for (limited) medical supplies, including antivirals, antibiotics and the first limited batches of pandemic vaccines that become available.

CHAPTER 8:

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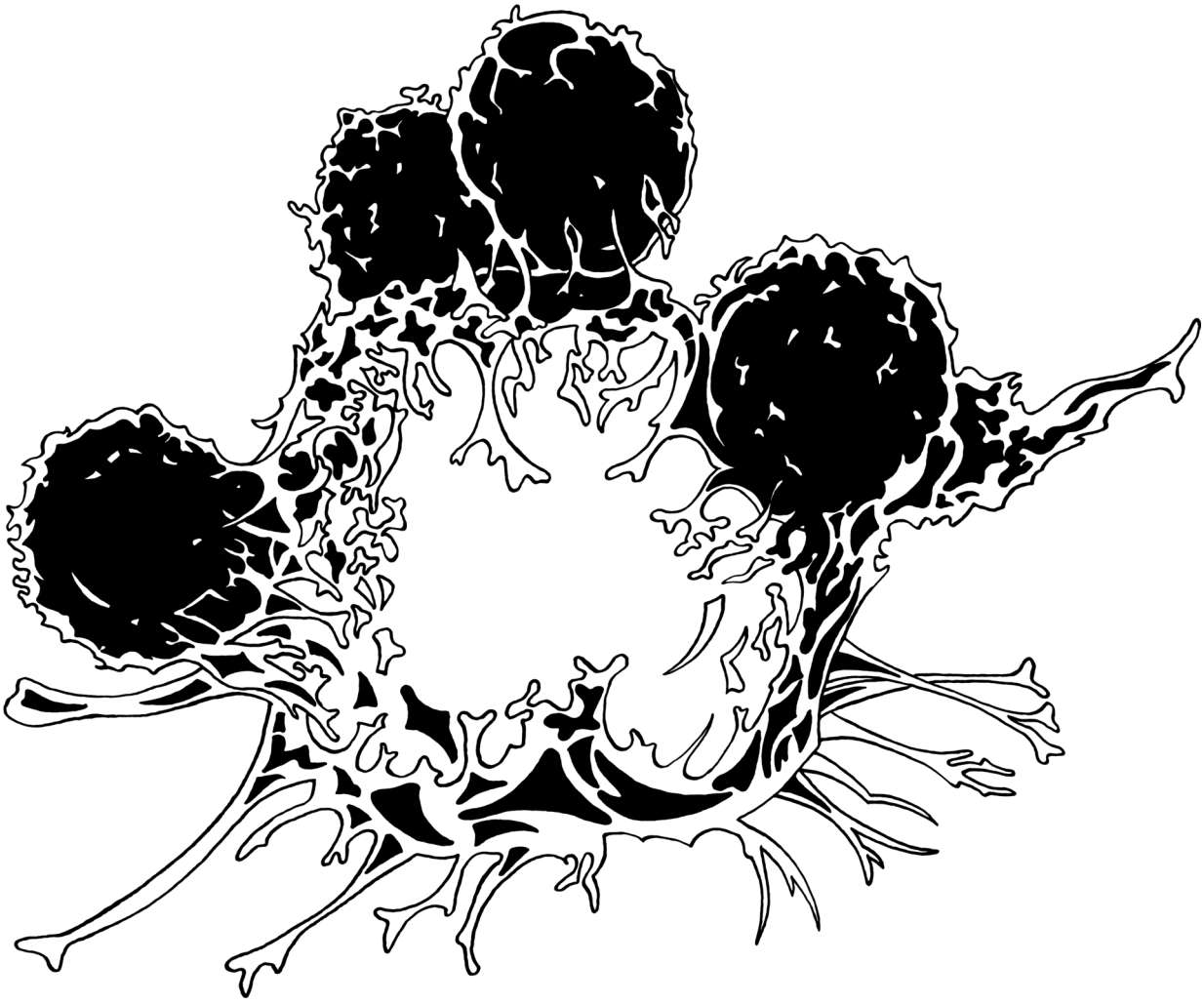

CHAPTER 9:

Nederlandse samenvatting

Influenzavirussen, ook wel griepvirussen genoemd, zijn een van de belangrijkste veroorzakers van luchtweginfecties bij mensen. Influenzavirussen worden onderverdeeld in vier verschillende types: A, B, C en D. De meeste influenzavirus-infecties bij mensen worden veroorzaakt door types A en B, dit zijn ook de influenzavirussen die worden besproken in dit proefschrift. Influenzavirussen kunnen verder van elkaar worden onderscheiden op basis van twee eiwitten aan de buitenzijde van het virusdeeltje, namelijk het hemagglutinine (HA) eiwit en het neuraminidase (NA) eiwit. Voor influenza A-virussen bestaan 18 verschillende HA eiwitten (H1-H18) en 11 verschillende NA eiwitten (N1-N11). De combinatie van deze twee eiwitten bepaalt tot welk subtype het influenza A-virus hoort, bijvoorbeeld A/H1N1, A/H3N2, A/H5N1 of A/H7N9. Bij influenza B-virussen worden geen subtypes onderscheiden. Door de genetische evolutie van de oppervlakte-eiwitten weer te geven in een (stam)boom, ook wel fylogenetische boom genoemd, kunnen er twee lijnen van influenza B-virussen worden onderscheiden, de B/Victoria-lijn en de B/Yamagata-lijn.
Influenzavirusinfecties komen vooral voor in de wintermaanden en zijn een jaarlijks terugkomend fenomeen, daarom worden deze influenzavirusinfecties ook wel de seizoensgriep genoemd. De seizoensgriep wordt veroorzaakt door influenza A-virussen van het A/H1N1- en A/H3N2-subtype en door influenza B-virussen van de B/Yamagata- en de B/Victoria-lijn. Het algemene ziektebeeld van deze seizoensgriep duurt ongeveer een week en gaat gepaard met klinische verschijnselen zoals verkoudheid, koorts, hoesten, spierpijn en hoofdpijn. De Wereldgezondheids-organisatie (World Health Organization; WHO) schat dat er jaarlijks drie tot vijf miljoen mensen een ernstige influenzavirusinfectie doormaken die gepaard kan gaan met een longontsteking; voor ongeveer 250.000-500.000 mensen is deze infectie fataal.

Het immuunsysteem speelt een belangrijke rol bij het detecteren en het opruimen van ziekteverwekkers zoals het influenzavirus. Gedurende een influenzavirus-infectie wordt specifieke afweer opgebouwd in de vorm van B-lymfocyten (B-cellen) en T-lymfocytes (T-cellen). B-cellen produceren antilichamen die onder andere gericht zijn tegen eiwitten aan de buitenzijde van het virusdeeltje, het HA en het NA eiwit. Antilichamen die gericht zijn tegen het HA kunnen voorkomen dat het virus een cel kan infecteren. Deze antilichamen worden ook wel neutraliserende antilichamen genoemd. De meeste van deze antilichamen zijn specifiek gericht tegen één subtype van het influenzavirus en zijn dus niet in staat andere subtypes te herkennen, laat staan te neutraliseren. Bovendien veranderen de HA en NA eiwitten langzaam, waardoor neutraliserende antilichamen die in het verleden zijn opgewekt tijdens een infectie met een bepaald subtype, niet in staat zijn een recente variant van ditzelfde subtype te herkennen. Dit proces van langzame verandering van de oppervlakte-eiwitten wordt ook wel antigenetische drift genoemd en resulteert in

jaarlijkse griepepidemieën.
Naast de productie van antilichamen door B-cellen, bestaat er ook cellulaire immuniteit die wordt gevormd door T-cellen. De virus-specifieke cytotoxische T-cellen, die worden besproken in dit proefschrift, zijn in staat geïnfecteerde cellen te herkennen en te elimineren. Tijdens de replicatiecyclus van het influenza-virus worden in geïnfecteerde cellen nieuwe virale eiwitten geproduceerd. Een deel van deze eiwitten wordt gebruikt om nieuwe virusdeeltjes te vormen, terwijl een ander deel in de cel wordt afgebroken tot kleine stukjes viraal eiwit, de zogenaamde peptiden. Een aantal van deze peptiden wordt gepresenteerd aan de buitenkant van de cel door middel van het Human Leucocyte Antigen (HLA). Wanneer een cytotoxische T-cel de combinatie van een virus-peptide (ook wel epitoop genoemd) en HLA herkent, doodt hij de geïnfecteerde cel om zo de productie van nieuwe virusdeeltjes te beperken. In tegenstelling tot de oppervlakte-eiwitten zijn de interne eiwitten, en dus ook de epitopen die door T-cellen worden herkend, sterk geconserveerd tussen de verschillende subtypes van het influenza A-virus. In het verleden is aangetoond dat cytotoxische T-cellen, die gericht zijn tegen een bepaald influenza A-virus subtype (Bijv. A/H3N2), in staat zijn om cellen die geïnfecteerd zijn met een ander subtype (Bijv. A/H1N1 of A/H5N1) te herkennen. Echter, tot op heden was het onbekend of deze kruis-herkenning ook plaats vindt tussen de twee lijnen van het influenza B-virus.
In hoofdstuk 2 wordt beschreven dat humane cytotoxische T-cellen, gericht tegen en gestimuleerd met een influenza B-virus van de B/Yamagata-lijn, in staat zijn om cellen geïnfecteerd met een influenza B-virus van de B/Victoria-lijn te herkennen en te doden, en omgekeerd. Het is dus goed mogelijk dat mensen die geïnfecteerd zijn met de ene lijn van het influenza B-virus een zekere mate van T-cel immuniteit hebben verworven die bescherming zou kunnen bieden tijdens een infectie met een virus van de andere influenza B-lijn.

Naast de jaarlijkse griepepidemieën wordt er zo nu en dan een influenza A-virus van een “nieuw” subtype geïntroduceerd in de humane populatie. De overdracht van een nieuw influenzavirus subtype afkomstig uit dieren (Bijv. vogels of varkens) op de mens wordt ook wel antigenetische shift genoemd. Het merendeel van de humane bevolking heeft echter geen antilichamen die gericht zijn tegen dit nieuwe subtype en zal onbeschermd zijn tegen infectie met dit nieuwe influenzavirus. Hierdoor zou het virus, als het overdraagbaar wordt van mens-op-mens, een nieuwe influenzapandemie kunnen veroorzaken. In de afgelopen eeuw hebben er vier van dit soort pandemieën plaats gevonden: in 1918 (Spaanse griep), 1957 (Aziatische griep), 1968 (Hong Kong griep) en zeer recentelijk in 2009 (Mexicaanse griep). De meeste slachtoffers vielen tijdens de Spaanse grieppandemie; in een jaar tijd stierven er wereldwijd tussen de 25 en 50 miljoen mensen aan de gevolgen van een infectie met dit nieuwe influenza A-virus subtype.

De meest recente pandemische dreiging komt uit China, waar sinds 2013 een vogelgriepvirus van het A/H7N9-subtype regelmatig infecties in mensen veroorzaakt. Tot nu toe zijn er 677 mensen geïnfecteerd, waarvan er 275 zijn overleden. Vooralsnog zijn deze virussen niet in staat om zich effectief van mens-op-mens te verspreiden, maar gevreesd wordt dat het virus zich in de toekomst weet aan te passen waardoor overdracht van mens-op-mens wel mogelijk wordt. Door het gebrek aan A/H7N9-specifieke antilichamen in de humane populatie, zullen mensen in hoge mate vatbaar zijn voor een infectie met dit virus. Het was echter onbekend of humane cytotoxische T-cellen, opgewekt tijdens een infectie met de A/H1N1 of A/H3N2 seizoens-influenzavirussen, wel in staat zouden zijn om A/H7N9 virus-geïnfecteerde cellen te herkennen en te elimineren. In het 3de hoofdstuk van dit proefschrift wordt aangetoond dat een groot aantal bekende influenza A-virus epitopen aanwezig is in het A/H7N9 influenzavirus. Daarnaast zijn humane cytotoxische T-cellen, gericht tegen en gestimuleerd met humane influenza A-virussen van het A/H1N1- en A/H3N2-subtype, in staat zijn om A/H7N9 virus-geïnfecteerde cellen te herkennen en te elimineren. Mensen die recentelijk een A/H1N1 of A/H3N2 infectie hebben doorgemaakt beschikken hoogst waarschijnlijk over T-cel immuniteit die een zekere mate van bescherming zou kunnen bieden tegen infecties met A/H7N9-virussen.

Een recente studie heeft aangetoond dat bestaande T-cel immuniteit in mensen kon worden gecorreleerd met een milder ziektebeeld tijdens de influenzapandemie van 2009. Het was echter niet bekend of mensen die over deze T-cel immuniteit beschikten recentelijk een seizoensgriep hadden doorgemaakt of dat deze cytotoxische T-cellen langere tijd aanwezig bleven in het lichaam. Het onderzoek beschreven in hoofdstuk 4 laat zien dat influenzavirus-specifieke cytotoxische T-cellen gedurende een lange periode kunnen worden aangetoond in het bloed van gezonde mensen. In de periode 1999 tot 2012 is er met een zekere regelmaat bloed van gezonde HLA-getypeerde vrijwilligers afgenomen. Door het plasma van deze gezonde vrijwilligers te controleren op de aanwezigheid van antilichamen gericht tegen influenza A-virussen die circuleerden tussen 1999 en 2012, kon worden bepaald of de vrijwilligers in deze periode een influenza A-virusinfectie hadden doorgemaakt. Hoewel het percentage influenzavirus-specifieke cytotoxische T-cellen verhoogd was in de periode na een influenzavirusinfectie, bleek deze snel terug te keren naar een vast laag percentage cytotoxische T-cellen, maar ze verdwenen nooit helemaal. Deze basis hoeveelheid bestond vooral uit een speciale selectie van geheugen T-cellen die erom bekend staan dat ze snel geactiveerd worden en/of vermenigvuldigen op het moment dat een persoon opnieuw een influenzavirusinfectie doormaakt. Hierdoor wordt het aannemelijk dat niet alleen personen die recent een seizoensgriep hebben doorgemaakt een zekere mate van T-cel immuniteit bezitten die kan kruis-reageren met een mogelijk pandemisch virus,

maar dat deze immuniteit ook aanwezig is in personen die gedurende een langere periode geen influenza A-virusinfectie hebben doorgemaakt.

Het is bekend dat influenza A-virussen in staat zijn te ontsnappen aan herkenning door influenzavirus-specifieke T-cellen door de betreffende epitopen te veranderen, ook wel muteren genoemd. Door deze veranderingen kan het epitoop niet meer aan het HLA binden, waardoor het epitoop niet meer gepresenteerd wordt op het celoppervlak van de geïnfecteerde cel; een andere mogelijkheid is dat de verandering ervoor zorgt dat het epitoop onherkenbaar wordt voor de epitoop-specifieke T-cellen. Hierdoor probeert het virus te voorkomen dat de geïnfecteerde cel wordt gedood voordat er nieuwe virusdeeltjes zijn geproduceerd.
Echter, niet alle epitopen zijn in staat te muteren. In sommige epitopen gaan mutaties ten koste van de functionaliteit van het eiwit waardoor de virussen niet meer optimaal kunnen repliceren. Het behoud van het epitoop weegt dan zwaarder dan het niet herkend worden door de cytotoxische T-cellen. Het influenza A-virus matrix 1 (M1) eiwit bevat een dergelijk geconserveerd epitoop, genaamd $M1_{58-66}$. Het is bekend dat mensen die het zogenaamde HLA-A*0201 tot expressie brengen (>40% van de blanke bevolking), dit epitoop aan het immuunsysteem presenteren en een sterke cytotoxische T-cel respons ontwikkelen tegen dit epitoop. Functionele beperkingen voorkomen dat het influenza A-virus het $M1_{58-66}$ epitoop kan veranderen, waardoor het niet lijkt te kunnen ontsnappen aan de specifieke cytotoxische T-cel respons. Toch blijkt uit onderzoek dat beschreven staat in hoofdstuk 5, dat het M1 eiwit afkomstig van een influenzavirus van het A/H5N1-subtype (een vogelgriepvirus) beter herkend wordt door $M1_{58-66}$-specifieke cytotoxische T-cellen dan een M1 eiwit dat afkomstig was van het humane A/H3N2 influenzavirus. Hoewel het epitoop zelf geconserveerd is tussen beide varianten van het M1 eiwit, bleek dat buiten het $M1_{58-66}$ epitoop een aantal kleine verschillen in de aminozuursequentie (de bouwstenen van een eiwit) voorkwamen. Door de aminozuren van het A/H5N1 M1 eiwit te introduceren in het A/H3N2 M1 eiwit, en omgekeerd, kon het verschil in herkenning door de $M1_{58-66}$-specifieke cytotoxische T-cellen deels worden omgedraaid. Humane influenza A-virussen lijken dus wel degelijk een strategie te hebben ontwikkeld om de presentatie van dit geconserveerd $M1_{58-66}$ epitoop te beperken, zonder het epitoop zelf te veranderen. Hierdoor worden cellen, die geïnfecteerd zijn met het humane A/H3N2 influenzavirus, minder goed herkend door $M1_{58-66}$-specifieke cytotoxische T-cellen, waardoor deze influenza-virussen langer kunnen “overleven” in de humane populatie. Mogelijk beïnvloeden aminozuren buiten het epitoop hoe het eiwit wordt afgebroken, waarbij de aminozuren op deze posities in humane virussen ervoor zorgen dat het $M1_{58-66}$ epitoop minder efficiënt wordt vrijgemaakt en dus in verminderde mate wordt gepresenteerd aan de buitenzijde van de geïnfecteerde cel. Opvallend is dat het influenza A-virus dat de relatief milde Mexicaanse grieppandemie veroorzaakte, een

M1 eiwit bevatte dat afkomstig was uit varkens. De aminozuren buiten het epitoop, die in deze studie worden geassocieerd met een verminderde herkenning door $M1_{58-66}$-specifieke cytotoxische T-cellen, waren niet aanwezig in het pandemische griepvirus van 2009 Dit is in tegenstelling tot de influenza A-virussen die de pandemieën van 1918, 1957 en 1968 veroorzaakten, waar deze aminozuren wel aanwezig waren. Mogelijk waren HLA-A*0201 positieve personen hierdoor beter beschermd tegen een ernstig ziektebeeld na infectie met het pandemische griep virus van 2009.

Het onderzoek dat beschreven staat in hoofdstuk 2 tot en met 5 toont aan dat cytotoxische T-cellen, in tegenstelling tot de meeste antilichamen, wel een bijdrage leveren aan kruis-beschermende immuniteit. Huidige griepvaccins zijn echter voornamelijk gericht op het induceren van antilichamen die specifiek gericht zijn tegen de influenzavirussen die op dat moment circuleren. Door continue antigenetische drift in deze seizoens-influenzavirussen moet het vaccin bijna jaarlijks worden aangepast om bescherming te kunnen blijven bieden tegen deze nieuwe varianten. Omdat de productie van een nieuw influenzavaccin een langdurig proces is, wordt ruim een half jaar voor het nieuwe griepseizoen bepaald welke virusstammen er in het nieuwe griepvaccin gebruikt zullen worden. In het geval van onverwacht snelle antigenetische drift van zowel influenza A- als B-virussen zal dit vaccin minder goed beschermen. In het geval van een nieuwe influenzapandemie zullen deze vaccins helemaal niet werken. Bovendien kan het in een pandemische situatie nog maanden duren voordat er voldoende effectieve vaccins op de markt komen. Idealiter zouden griepvaccins gericht tegen de seizoensgriep ook in staat moeten zijn een bredere immuun respons op te wekken die kruis-bescherming biedt in het geval van onverwacht snelle antigenetische drift of tegen een mogelijk pandemisch influenzavirus. In hoofdstuk 6 werd onderzocht of het toevoegen van een adjuvans, een hulpstof die aan een vaccin kan worden toegevoegd om de immuunrespons te versterken, in staat is om de immuunrespons ook te verbreden. In deze studie werden muizen twee keer gevaccineerd met het standaard geïnactiveerde griepvaccin, al dan niet in combinatie met het nieuwe G3/DT adjuvans. Uit deze studie bleek dat alleen muizen die gevaccineerd waren met een vaccin dat was gecombineerd met het G3/DT adjuvans tot op zekere hoogte beschermd waren tegen een influenzavirus stam die niet in het vaccin vertegenwoordigd was. Deze kruis-bescherming kon worden toegeschreven aan de door G3/DT geïnduceerde cytotoxische T-cel respons, die afwezig was in muizen die enkel met het griepvaccin zelf werden gevaccineerd.

Samenvattend, tonen de studies beschreven in dit proefschrift aan dat virus-specifieke cytotoxische T cellen kruis-reageren met verschillende influenza A- of B-virussen en een bijdrage leveren aan kruis-beschermende immuniteit. Deze

T-cel-gemedieerde immuniteit zou de ernst van een ziekte veroorzaakt door een nieuw influenzavirus kunnen reduceren, zoals ook beschreven is voor het pandemische griepvirus van 2009. Overigens worden potentieel pandemische influenza A-virussen van aviaire oorsprong beter herkend door $M1_{58-66}$-specifieke T-cellen dan humane influenza A-virussen. Verschillen in aminozuren buiten het epitoop waren hier verantwoordelijk voor en wijzen op een adaptatiestrategie van humane influenzavirussen om te ontsnappen aan herkenning door deze $M1_{58-66}$-specifieke T-cellen. Het gebruik van vaccins die niet alleen gericht zijn op de inductie van virus-specifieke antilichamen, maar ook op het induceren van virus-specifieke cytotoxische T-cellen, verdient de voorkeur. Het toevoegen van bepaalde adjuvantia, zoals G3/DT, aan bestaande vaccinpreparaten zou de inductie van virus-specifieke T-cellen bevorderen. Dergelijke vaccins zouden niet alleen in staat zijn bescherming te bieden tegen de jaarlijkse griepvirussen, maar ook tegen varianten met onvoorspelbare antigenetische drift en potentieel pandemische influenzavirussen van nieuwe subtypes.

CHAPTER 10:

Dankwoord

–Time flies when you are having fun–

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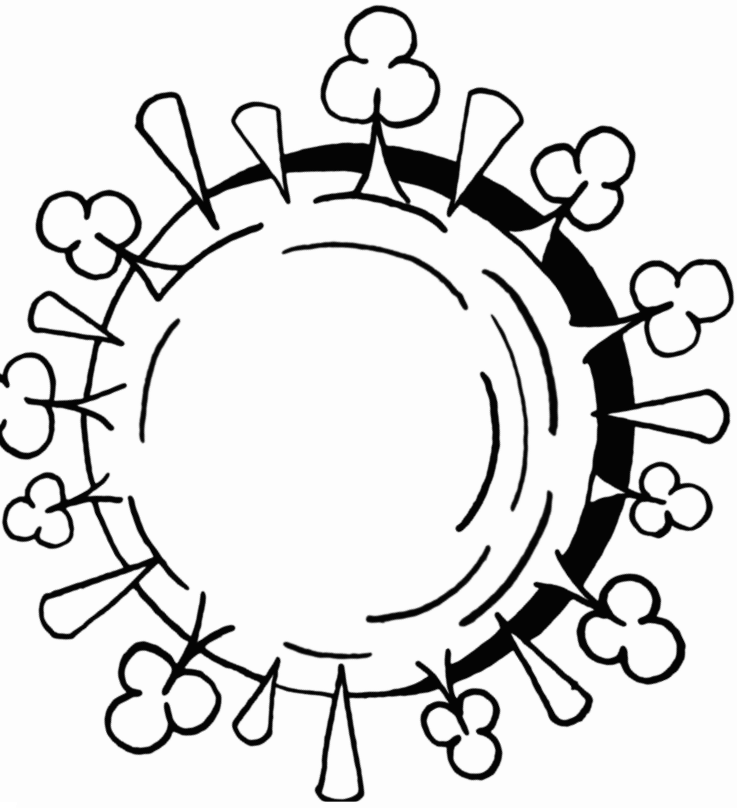

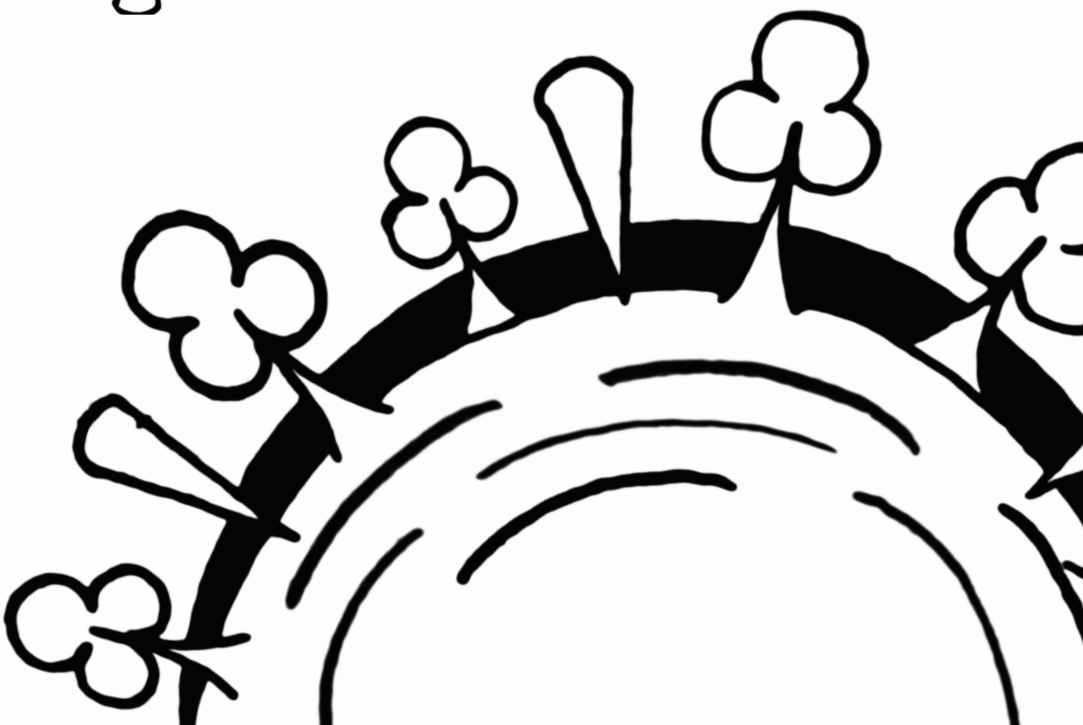

CHAPTER 11:

About the author

Curriculum Vitae

PhD portfolio

Publications

Curriculum Vitae

Carolien van de Sandt was born on February 22, 1987 and lived in a small town called Eibergen, The Netherlands. In 2005 she finished high school (VWO) at Marianum in Groenlo and started to study Biomedical Sciences at Leiden University. She soon found out that her primary interests were in the field of virology and immunology. The research of her bachelor internship focused on developing a Yellow Fever Virus (vaccine strain) expressing the Lassa GP1 and GP2 proteins under the supervision of Dr. Peter Bredenbeek, at the department of Medical Microbiology of the Leiden University Medical Center. During her first master's internship she explored the possibilities of using artificial antigen presenting cells in expanding antigen-specific $CD8^+$ regulatory T cells under the supervision of Dr. Miranda Dierselhuis and Prof. dr. Els Goulmy at the department of Immunohematology and Bloodtransfusion of the Leiden University Medical Center. Her graduation internship under, the supervision of Dr. Mark Melchers and Dr. Rogier Sanders, at the Laboratorium for Experimental Virology of the Amsterdam Medical Center, focused on enhancing the immunogenicity of the HIV-1 Envelope protein by developing an octadecameric HIV-1 envelope construct that was fused to CD40L. Her research proposal for this graduation internship was awarded with the Heart of Biomedical Science prize. During her Master study, Carolien was a member of the Biomedical Sciences Symposium organizing committee. She received her Master of Science degree in January 2011 and soon thereafter she started as a PhD student at the department of Viroscience of the Erasmus Medical Center in Rotterdam under the supervision of Prof. Dr. Rimmelzwaan and Prof. Dr. Osterhaus. The focus of her research was the balance of cross-reactivity and viral evasion of the influenza virus-specific $CD8^+$ T cell response and has resulted in the present thesis. She will continue her work at the department of Viroscience focusing on the extra-epitopic amino acid residues that are involved in viral evasion of the influenza virus-specific $CD8^+$ T cell response.

PhD portfolio

Name PhD student	: Carolien Emma van de Sandt
Research department	: Viroscience, Erasmus MC
Research School	: Post-graduate Molecular Medicine
PhD period	: 2011-present
Promotors	: Prof. Dr. G.F. Rimmelzwaan Prof. Dr. A.D.M.E. Osterhaus

In-depth courses

- Course in Adobe InDesign CS5 (MolMed, Erasmus MC) 2015
- Course in Grant writing (Erasmus MC) 2015
- Couse in Comparative Pathology (MolMed and department of Viroscience, Erasmus MC) 2013
- Course in Virology (MolMed and department of Viroscience, Erasmus MC) 2012
- Course in Submandibular bleeding of mice (Erasmus Dierexperimenteel Centrum, Erasmus MC) 2012
- Course in Immunology (Leiden Institute for Immunology (LIFI), LUMC) 2011
- Course in Adobe Photoshop and Illustrator CS5 (MolMed, Erasmus MC) 2011
- Internal and external presentations (twice a week at the department of Viroscience, Erasmus MC)
- Frequent attendance T Cell Consortium Meeting (once a month, Erasmus MC)

(Inter)national conferences

- 7th Orthomyxovirus Research Conference, Toulouse, France (Sept 16-18) 2015
- 19th Molecular Medicine Day, Rotterdam, The Netherlands (March 19) 2015
- NVVI, Lunteren, The Netherlands (March 26-27) 2015
- Dutch Annual Virology symposium, Amsterdam, The Netherlands (March 6) 2015
- Nationaal Ebola actualiteitencafé, Rotterdam, The Netherlands (Oct 20) 2014
- 5th European Influenza Conference (ESWI), Riga, Letland (Sept 14-17) 2014
- Structure, Receptor binding and Neutralization of HIV, HCV and Influenza virus, Amsterdam The Netherlands (March 20) 2014
- Dutch Annual Virology symposium, Amsterdam, The Netherlands (March 7) 2014
- 18th Molecular Medicine Day, Rotterdam, The Netherlands (Feb 20) 2014
- NVVI winterschool, Noordwijkerhout, The Netherlands (Dec 18-19) 2013
- Options for the Control of Influenza VIII, Cape Town, South Africa (Sept 5-9) 2013
- Dutch Annual Virology symposium, Amsterdam, The Netherlands (March 8) 2013
- 17th Molecular Medicine Day, Rotterdam, The Netherlands (Feb 13) 2013
- The Vaccines and Vaccination Symposium, Leiden, The Netherlands(Oct 10) 2012
- 6th Orthomyxovirus Research Conference, Bromont, Canada (Sept 19-22) 2012

- Advances in Immunotherapy and Immunomonitoring; It's all about T cells, Amsterdam, The Netherlands (Sept 13) 2012
- NVVI, Lunteren, The Netherlands (March 29-30) 2012
- Dutch Annual Virology symposium, Amsterdam, The Netherlands (March 2) 2012
- 16th Molecular Medicine Day, Rotterdam, The Netherlands (Feb 29) 2012
- NVVI winterschool, Noordwijkerhout, The Netherlands (Dec 14-15) 2011
- Ruysch lecture T cell Immunity (Dec 5) 2011
- Joint Vaccine Meeting NOW WG Vaccines Minisymposium: New developments in vaccine adjuvants, Utrecht, The Netherlands 2011

Poster presentations

- Cross-reactivity of influenza virus-specific human cytotoxic T lymphocytes 2015
  19th Molecular Medicine Day, Rotterdam, The Netherlands (March 19)
- Human Cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus 2014
  18th Molecular Medicine Day, Rotterdam, The Netherlands (Feb 20)
- Viral evasion from recognition by influenza virus specific Cytotoxic T Lymphocytes 2012
  16th Molecular Medicine Day, Rotterdam, The Netherlands (Feb 29)

Oral presentations

- A novel strategy of influenza A viruses to evade recognition by influenza A virus-specific Cytotoxic T lymphocytes 2015
  7th Orthomyxovirus Research Conference, Toulouse, France (Sept 16-18)
- Cross-reactivity of influenza virus-specific human cytotoxic T lymphocytes (Elevator Pitch) 2015
  19th Molecular Medicine Day, Rotterdam, The Netherlands (March 19)
- Cross-reactivity of influenza A and B virus-specific human Cytotoxic T Lymphocytes 2015
  Dutch Annual Virology symposium, Amsterdam, The Netherlands (March 6)
- Human Cytotoxic T Lymphocytes directed to influenza B viruses cross-react with viruses of two phylogenetic lineages 2014
  5th European Influenza Conference (ESWI), Riga, Letland (Sept 14-17)
- Human Cytotoxic T Lymphocytes directed at human influenza A virus cross-react with the newly emerging H7N9 virus 2013
  Options for the Control of Influenza VIII, Cape Town, South Africa (Sept 5-9)
- Viral evasion from recognition by influenza virus-specific Cytotoxic T lymphocytes 2012
  6th Orthomyxovirus Research Conference, Bromont, Canada (Sept 19-22)

Grants and Awards

- 7th Orthomyxovirus Research Conference travel grant 2015
- Award for best poster presentation, 19th Molecular Medicine Day 2015
- ESWI Young Scientist travel grant, 5th European Influenza Conference 2014
- Promising Investigator Scholarship, Options for the Control of Influenza VIII 2013
- 6th Orthomyxovirus Research Conference travel grant 2012

Chair and Teaching activities

- Coach Viruskenner 2014-15
- Glovebox training Dutch Mobile Labs 2014-15
- Supervision of MSc student labrotations 2011-14
- Co-chair of the Innate and Adaptive Immunology workshop at Options for the Control of influenza VIII 2013

Publications

1. Altenburg AF*, **van de Sandt CE***, Li BWS, MacLoughlin R, De Gruyter HLM, van Amerongen G, van Run P, Pronk MR, Nieuwkoop NJ, Vogelzang-van Trierum SE, Hendriks RW, Sutter G, Rimmelzwaan GF, de Vries RD.
In vivo tropism of modified vaccinia virus ankara expressing green fluorescent protein after administration in the muscles or airways. *Manuscript in preparation*

2. **van de Sandt CE**, Kreijtz JHCM, Geelhoed-Mieras MM, Nieuwkoop NJ, Spronken MIJ, van de Vijver DAMC, Fouchier RAM, Osterhaus ADME, Rimmelzwaan GF.
Extra-epitopic residues determine differential recognition of influenza A viruses by $M1_{58-66}$ epitope-specific T cells. *J Virol, in press*

3. **van de Sandt CE**, Bodewes R, Rimmelzwaan GF, de Vries RD.
Influenza B viruses: not to be discounted. 2015. Future Microbiol. 10: 1447-65

4. **van de Sandt CE**, Dou YY, Vogelzang-van Trierum SE, Westgeest KB, Pronk MR, Osterhaus ADME, Fouchier RAM, Rimmelzwaan GF, Hillaire MLB.
Influenza B virus-specific $CD8^+$ T lymphocytes strongly cross-react with viruses of the opposing influenza B lineage. 2015. J Gen Virol. 96(8):2061-73.

5. **van de Sandt CE**, Rimmelzwaan GF.
Immunodominant responses to influenza virus $M1_{58-66}$ epitope: Stealth or protection? 2015. Proc Natl Acad Sci USA. 12(19):E2417.

6. **van de Sandt CE**, Hillaire MLB, Geelhoed-Mieras MM, Ostrhaus ADME, Fouchier RAM, Rimmelzwaan GF.
Human influenza A virus-specific $CD8^+$ T-cell response is long-lived. 2015. J Infect Dis. 212(1):81-85.

7. Jong WSP, Daleke-Schermerhorn MH, Vikström D, ten Hagen-Jongman CM, de Punder K, van der Wel NN, **van de Sandt CE**, Rimmelzwaan GF, Follmann F, Agger EM, Andersen P, de Gier JW, Luirink J.
An autotransporter display platform for the development of multivalent recombinant bacterial vector vaccines. 2014. Microb Cell Fact. 12:162-176.

8. **van de Sandt CE**, Kreijtz JHCM, Geelhoed-Mieras MM, Vogelzang-van Trierum SE, Nieuwkoop NJ, van de Vijver DAMC, Fouchier RAM, Osterhaus ADME, Morein B, Rimmelzwaan GF.
Novel G3/DT adjuvant promotes the induction of protective T cells responses after vaccination with a seasonal trivalent inactivated split-virion influenza vaccine. 2014. Vaccine. 32(43):5614-5623.

9. **van de Sandt CE***, Kreijtz JHCM*, de Mutsert G, Geelhoed-Mieras MM, Hillaire MLB, Vogelzang-van Trierum SE, Osterhaus ADME, Fouchier RAM, Rimmelzwaan GF.
Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus. 2014. J Virol. 88(3):1684-1693.

10. **van de Sandt CE**, Kreijtz JHCM, Rimmelzwaan GF.
Evasion of influenza A viruses from innate and adaptive immune responses. 2012. Viruses. 4(9):1438-1476.

11. Melchers M, Matthews K, de Vries RP, Eggink D, van Montfort T, Bontjer I, **van de Sandt C**, David K, Berkhout B, Moore JP, Sanders RW.
A stabilized HIV-1 envelope glycoprotein trimer fused to CD40 ligand targets and activates dendritic cells. 2011. Retrovirology. 8:48-63

**: Authors contributed equally to the study*