

Review Article

Induction of Virus-Specific Cytotoxic T Lymphocytes as a Basis for the Development of Broadly Protective Influenza Vaccines

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There is considerable interest in the development of broadly protective influenza vaccines because of the continuous emergence of antigenic drift variants of seasonal influenza viruses and the threat posed by the emergence of antigenically distinct pandemic influenza viruses. It has been recognized more than three decades ago that influenza A virus-specific cytotoxic T lymphocytes recognize epitopes located in the relatively conserved proteins like the nucleoprotein and that they cross-react with various subtypes of influenza A viruses. This implies that these CD8⁺ T lymphocytes may contribute to protective heterosubtypic immunity induced by antecedent influenza A virus infections. In the present paper, we review the evidence for the role of virus-specific CD8⁺ T lymphocytes in protective immunity against influenza virus infections and discuss vaccination strategies that aim at the induction of cross-reactive virus-specific T-cell responses.

1. Introduction

Every year, influenza A viruses (IAVs) cause epidemic outbreaks of respiratory tract infection resulting in excess morbidity and mortality. Especially individuals with certain underlying medical conditions and the elderly are at risk for complications of influenza. Therefore, it is recommended to vaccinate these individuals against influenza annually.

Currently used vaccines largely aim at the induction of antibodies directed to viral glycoproteins, in particular the hemagglutinin (HA). These antibodies neutralize the virus by preventing viral attachment to host cells and are generally considered the main correlate of protection against influenza virus infection [1]. Therefore, assessing postvaccination HA-specific antibody titers is used as surrogate marker for vaccine efficacy compliant with EMEA and FDA guidelines [2, 3].

However, seasonal influenza viruses continuously accumulate amino acid substitutions in the antigenic sites of the

HA molecules and consequently display considerable antigenic drift.

This allows currently circulating influenza viruses to escape from the neutralizing activity of antibodies induced by previous infections or vaccination and necessitates updating the vaccine regularly to match recent epidemic strains.

Occasionally, novel strains of influenza A viruses are introduced with HA molecules that are antigenically distinct from seasonal influenza A viruses including those of a novel subtype. Seasonal influenza vaccines are not protective against these new viruses, which may spark a pandemic outbreak of influenza and against which specific vaccines need to be developed. The pandemic of 2009 caused by influenza A/H1N1 viruses of swine origin painfully highlighted that the development of a matching vaccine is a time consuming process, and, in many countries, vaccines became available after the peak of pandemic [4, 5].

For these reasons, there is considerable interest in the development of more broadly protective influenza vaccines

that ideally would afford broad protection against various subtypes of influenza A viruses [6, 7] and/or antigenic drift variants within a subtype.

It has been well established that infection with influenza A virus can induce a certain degree of protective immunity to infection with other influenza A viruses of unrelated subtypes (heterosubtypic immunity) (for review see [8]). Elucidation of the correlates of protection of this type of immunity may aid the development of more universal vaccines. Since different subtypes of influenza A virus are defined by the absence of serological cross-reactivity, it is unlikely that antibodies to HA or neuraminidase (NA) contribute to this type of infection-induced immunity to a great extent. However, recently antibodies have been identified specific for epitopes located in the stem region of the HA molecule, displaying broad reactivity and broad neutralizing activity against several different influenza A viruses of different HA subtypes [9–16]. HA-stem-based vaccines may be a promising venue for the development of broadly protective vaccines. Other vaccine candidates aiming at the induction of cross-protective antibodies may include those based on the M2 protein [17–21]. The induction of M2-specific antibodies after infection is rather inefficient. Furthermore, several studies have shown that postinfection serum does not afford protection against a heterosubtypic strain of influenza A virus, whereas virus-specific T cells do [22]. Nevertheless, induction of M2-specific antibodies after M2 hyperimmunization does afford heterosubtypic immunity. However, it was shown that the protection mediated by vaccine-induced M2 antibodies was weak and could not prevent infection of mice. The mechanism of protection was based on antibody-dependent cell cytotoxicity (ADCC) [23].

Since the majority of virus-specific T cells, and in particular CD8⁺ cytotoxic T lymphocytes (CTL), are directed against relatively conserved viral proteins like the nucleoprotein (NP) and the matrix 1 protein (M1), it was already suggested three decades ago that virus-specific CTLs contribute to heterosubtypic immunity [24, 25]. In the present paper, we review the evidence that influenza virus-specific T cells contribute to (cross-)protective immunity and discuss vaccine formulation that can induce virus-specific CTL.

2. CTLs Contribute to Heterosubtypic Immunity

The most important mode of action of virus-specific CTL is recognition and elimination of virus-infected cells. This way, the production of progeny virus is prevented. Thus, the presence of preexisting T-cell immunity results in more rapid clearance of virus infections. Key for heterosubtypic immunity is that CTLs are cross-reactive and recognize epitopes shared by influenza A viruses of different subtypes. The effector functions of CTLs that are responsible for the elimination of virus-infected cells include the release of perforin and granzyme from their granules and Fas/FasL interactions with infected target cells. In addition, upon activation virus-specific CD8⁺ T cells can produce a variety of different cytokines including IFN- γ and TNF- α . It was shown

that virus-specific CTLs, through their receptor recognize viral peptides, which are generated by the endogenous route of antigen processing and that are ultimately presented by MHC class I molecules on the surface of antigen-presenting cells or virus-infected cells [26, 27]. For the efficient induction of virus-specific CTLs, it is required that the antigen is present in the cytosol of antigen-presenting cells where antigen processing takes place.

Influenza virus-specific CTL can recognize epitopes that are shared by different subtypes of influenza A virus. Indeed, it was shown that a large proportion of mouse and human CTLs induced after infection with influenza A virus were directed against the relatively conserved NP and M1 protein [29, 31, 37–39]. This raised the expectation that these cells contribute to cross-protection against viruses of different subtypes.

Many studies have been performed to demonstrate the cross-reactivity of influenza virus-specific CTLs and their role in heterosubtypic immunity. The outcomes of these studies are summarized in Table 1.

2.1. Evidence for Cross-Reactivity of CTL In Vitro. Early evidence for the intersubtypic cross-reactivity of CTL was described by Zweerink et al. [28, 40], who demonstrated that mouse CTL specific for influenza virus of the H2N1 subtype could lyse target cells infected with virus of the H3N2 subtype.

Also with other combinations of subtypes, the cross-reactive nature of virus-specific CTL was confirmed (Table 1). For example, it was shown that in healthy individuals, with a history of infection with seasonal influenza virus, memory CD8⁺ T cells were present in the blood that cross-reacted with highly pathogenic H5N1 virus [32–34]. The presence of cross-reactive CTL may afford a certain degree of protection against infection with these viruses, which still constitute a pandemic threat. It is of interest to note that especially younger individuals are at risk for severe disease and fatal outcome of influenza H5N1 infection [41]. It is tempting to speculate that younger individuals less likely have been exposed to seasonal influenza A viruses of the H3N2 or H1N1 subtype and, thus, have not mounted a CTL response to these viruses. Therefore, they may be more susceptible to infection with a virus of an alternative subtype. However, it cannot be ruled out that other factors play a role in the observed disproportionate age distribution of severe H5N1 cases. Furthermore, CTL obtained from healthy subjects before the pandemic of 2009 displayed cross-reactivity with the pandemic 2009 pH1N1 virus [35, 36], which may have afforded a certain degree of protection against this virus.

2.2. Evidence for the Role of CTL in Protection against Infection. Evidence for the role of virus-specific CTL in protection against influenza virus infection predominantly stems from animal models (Table 2). Using various combinations of influenza A virus subtypes, it was demonstrated that CTL responses induced after a primary infection with influenza virus either correlated with protection against challenge infection with a virus of another subtype or were responsible

TABLE 1: Evidence for cross-reactivity of influenza virus-specific CTL.

Subtype		Species	Comments	Ref.
Priming subtype	Cross-reaction with			
H2N1	H3N2	Mouse	Cross-reactivity confirmed in Cr-release assays using cultured splenocytes of primed mice	[28]
H1N1	H2N2 H3N2	Mouse	Cross-reactivity confirmed in Cr-release assays using splenocytes of primed mice	[24]
H3N2	H1N1	Mouse	Target cells expressing NP from H1N1 recognized by cultured splenocytes from primed mice	[29]
H3N2	H1N1	Mouse	Cross-reactive cultured splenocytes recognize inner proteins of influenza A virus	[30]
H3N2	H1N1	Human	Cross-reactive CTLs recognize NP, M1, or PB2	[31]
Seasonal influenza	H5N1	Human	Cross-reactive CTLs were detected in the blood of healthy human subjects not exposed to H5N1 virus	[32–34]
pH1N1	H1N1	Human	T cells specific for the NP418 epitope induced by the 2009 pH1N1 cross-react with the 1918-H1N1 variant but not with contemporary variants	[35]
Seasonal influenza	2009 pH1N1	Human	Cross-reactive CTLs were detected in the blood of healthy human subjects not exposed to 2009 pH1N1 virus	[36]

for protective immunity [8, 42–47]. The full extent of cross-reactivity of human CTL is not known. However, since the conserved proteins like the NP, M1, and the polymerase proteins display a high degree of sequence homology, it is assumed that the extent of cross-reactivity between different subtypes of influenza A virus is substantial. This is exemplified by demonstrating that human CTLs directed to human influenza A viruses of the H3N2 subtype cross-react considerably with avian influenza A viruses of the H5N1 subtype [42].

By adoptive transfer or depletion of virus-specific CD8⁺ T cells, it was confirmed that these cells contributed to protective heterosubtypic immunity [22, 48–50, 52]. Indeed, the adoptive transfer of virus-specific CTLs to naïve recipient mice had a beneficial effect on the course of subsequent challenge infections. It was shown that transfer of CTL from mice infected with seasonal H3N2 virus protected recipient mice against challenge infection with 2009 pH1N1 virus [22]. Also chickens that received CTL from chickens infected with H9N2 virus were protected against subsequent challenge infection with highly pathogenic H5N1 virus [56]. Also, depletion of CD8⁺ T cells prior to challenge infection confirmed that these cells contribute to heterosubtypic immunity. Primed mice or chickens, from which CTL were depleted, had higher lung virus titer, developed more severe disease, and displayed higher mortality rates after challenge infection than control animals [54, 55, 57, 59].

There is little evidence that CTLs contribute to heterosubtypic immunity in humans. The first and, to our knowledge, the only evidence for this was described by McMichael et al. They demonstrated that in experimentally infected individuals, virus-specific cytotoxicity inversely correlated with the extent of virus shedding in the absence of antibodies specific for the H1N1 strain that was used for infection [38].

There is, however, epidemiological evidence that indicate that prior exposure to influenza viruses is inducing protective immunity against a heterosubtypic strain of influenza [60].

People, who experienced symptomatic influenza caused by infection with influenza viruses of the H1N1 subtype, were partially protected from infection with the pandemic H2N2 viruses in 1957 [60]. A possible correlation with the presence of virus-specific CTL-mediated immunity was not studied. More circumstantial evidence is based on the observation that the ratio between synonymous and nonsynonymous (Ds/Dn) mutations in the NP gene is lower in CTL epitope sequences than in the rest of the protein. This also provides indirect evidence that CTLs exert antiviral activity in humans at the population level [61] and indicates that CTL epitopes are under selective pressure. Indeed, a number of amino acid substitutions that were observed in CTL epitopes during the evolution of influenza A/H3N2 viruses were associated with escape from recognition by virus-specific CTL [61–65]. Examples include the R384G substitution at the anchor residue of the HLA-B*2705 restricted NP_{383–391} epitope and amino acid substitutions at T-cell receptor contact residues of the HLA-B*3501 restricted NP_{418–426} epitope. In both cases, the amino acid substitutions affected the *in vitro* human influenza virus-specific CTL response significantly [66, 67].

Of interest, the R384G substitution alone was detrimental to viral fitness and was only tolerated in the presence of two functionally compensating mutations [68, 69].

Thus, apparently, the virus has the capacity to overcome functional constraints in order to evade T-cell immunity. The rapid fixation of the R384G substitution could be explained by strong bottle-neck and founder effects at the population level in a theoretical model [70]. Although CTL epitopes, can thus display variability allowing the virus to escape from recognition by CTL specific for these epitopes, other epitopes remain fully conserved including the immunodominant M1_{58–66} epitope that is restricted by HLA-A*0201, which has a high prevalence in most countries. For this and some other conserved epitopes it was demonstrated that also functional constraints may play a role in limiting the virus

TABLE 2: Evidence for a role of CTLs in cross-protective immunity against influenza virus infections.

Priming	Subtype used	Challenge	Species	Experiment	Comments	Ref
H1N1		H1N1	Mouse	Adoptive transfer	Splenocytes depleted from CD8 ⁺ T cells failed to protect against infection.	[25]
H1N1 or H3N2		H1N1 or H3N2	Mouse	Adoptive transfer	T cells afford protection against infection with heterosubtypic strain.	[48]
H3N2		H3N2	Nude mouse	Adoptive transfer	Immune spleen cells from Balb-c mice mediated more rapid clearance of virus infection and correlated with cytolytic activity.	[49]
H3N2		H1N1				
H3N2		H3N2	Mouse	Adoptive transfer	Mouse NP-specific CTL clone afforded protection.	[50]
		H2N2				
NP from H3N2		H1N1	Mouse	Adoptive transfer	NP priming promoted recovery and was attributed to cross-reactive CTLs.	[51]
H2N2		H1N1	Mouse	Adoptive transfer	Cross-reactive CTL clones conferred protection.	[52]
H3N2		H3N2	Mouse	Adoptive transfer	NP-specific CTL clone reduced lung virus titers and mortality rates.	[53]
H1N1		H3N2	Mouse	Depletion of T cells	CTLs conferred heterosubtypic immunity and reduced virus titers in the respiratory tract.	[54]
H3N2		H1N1	Mouse		Protection correlated with CTL response.	[45]
H9N2		H5N1	Mouse		Protection correlated with CTL response to NP or PB2.	[46]
H1N1		H2N2	Mice KO for Ig or γ/δ T cells	Acute depletion of CD8 ⁺ T cells	In absence of Ig or γ/δ T cells, CTLs conferred protection.	[44]
H1N1		H3N2	Mouse		Protection correlated with CTL response to NP.	[43]
H3N2		H5N1	Mouse		Protection correlated with CTL response to NP and PA.	[42]
Gamma-irradiated viruses		H1N1	Mouse	Adoptive transfer of CD8 ⁺ T cells	CTL but not B cells afforded protection.	[36]
H3N2		2009 pH1N1	Mouse	Depletion of CD8 ⁺ T cells	CTL conferred heterosubtypic immunity to 2009 pH1N1 virus.	[55]
H3N2		2009 pH1N1	Mouse	Adoptive transfer of CD8 ⁺ and CD4 ⁺ T cells	CTL conferred heterosubtypic immunity to 2009 pH1N1 virus.	[22]
H9N2		H5N1	Chicken	Adoptive transfer of CD8 ⁺ T cells	CTLs reduced mortality rates.	[56]
H9N2		H5N1	Chicken	Depletion	CTLs reduced mortality rates.	[57]
H3N2		H5N1	Ferret		Protection correlated with CTL response.	[58]
		H1N1	Human		CTL activity correlated with protection in absence of antibodies.	[37]

to escape efficiently from recognition by CTL to these highly conserved epitopes [71, 72]. Thus, influenza virus CTL epitopes are either conserved, display variation at non-anchor residues, or lose their anchor residues at the cost of viral fitness, which need to be functionally compensated by the accumulation of mutations.

3. Considerations for Vaccine Development

A large number of peptides are generated during processing of viral proteins in infected cells, but only some of these peptides are ultimately presented by major histocompatibility complex class I molecules and recognized by specific CTL. The hierarchy of CTL responses is called immunodominance [73–75] and has been demonstrated in animal models [76] and humans [77].

In mice it was shown that the hierarchy of primary and secondary CTL responses differ [76, 78, 79]. Since some CTL epitopes are more dominant than others, also the HLA usage of the CTL response is dependent on the repertoire of viral epitopes. For this reason, the HLA usage of the CTL response to influenza A virus is different from that to influenza B virus [80]. Immunodominance also complicates the analysis of CTL responses induced by vaccination or infection. Assessing the response to a single epitope is not fully informative without knowing its relative immunodominance. In addition, it has been shown that the response to a single epitope can be influenced by other non-corresponding HLA alleles [77, 81].

The HLA haplotype dictates which epitopes can be presented and recognized and determines the magnitude of the virus-specific CTL response. For example, the immunodominant M1_{58–66} epitope is only recognized in HLA-A*0201 positive subjects, and, in these individuals, the overall CTL response to influenza A virus is higher than in HLA-A*0201 negative subjects that are matched for the remaining HLA alleles [77].

Thus, both immunodominance and HLA restriction of CTL responses should be taken into account when assessing the ability of candidate vaccines to induce virus-specific CTL responses.

For the efficient induction of CTL responses, it is critical that viral antigens enter the endogenous route of antigen processing. To achieve this, viral proteins need to be delivered in the cytosol of antigen presenting-cells, where degradation of these proteins by the proteasome takes place. The peptides that are generated are then transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum where binding of antigenic peptides with their corresponding MHC class I molecules can take place. These MHC class I/peptide complexes are subsequently transported to the cell membrane for recognition by virus-specific CD8⁺ T lymphocytes. The cytosolic delivery of viral proteins by vaccine preparations can be achieved by using live (attenuated) virus, viral vectors, or expression from plasmid DNA, which allow *de novo* synthesis of viral proteins in the infected cells. Alternatively, particulate antigen presentation forms can be used which can translocate viral proteins into

the cytosol directly or through endosomal degradation of the exogenous viral proteins [82].

In addition to CD8⁺ T cells, CD4⁺ T cells have been shown to contribute to heterosubtypic immunity [54, 55]. The relationship between CD4⁺ and CD8⁺ T cells has been studied extensively, and it seems that memory CD8⁺ T cells are impaired in the absence of memory CD4⁺ T cells leading to increased cell death and decreased secondary T-cell response [83]. Thus, it is imperative that vaccines also induce adequate virus-specific CD4⁺ T-helper cell responses in addition to CD8⁺ T-cell responses. Of interest, also CD4⁺ T cells specific for seasonal influenza A viruses display cross-reactivity with influenza A viruses of different subtypes including 2009 pH1N1 [84] and H5N1 [32, 34, 85].

Other cells of the adaptive immune system may play a role in heterosubtypic immunity against influenza A viruses. Some studies have indicated that B cells and mucosal antibodies play a role in heterosubtypic immunity [23, 44, 86–90]. However, we and others were able to adoptively transfer heterosubtypic immunity with T cells but not with B cells to naïve recipient mice (Table 2). Of interest, CD4⁺ T cells are necessary to promote the protective effect of virus-specific CD8⁺ T cells [22]. However, since this special issue focuses on CD8⁺ T cells we did not discuss the role of other immune cells extensively. Also cells of the innate immune system (like NK cells and macrophages) have protective efficacy; however, since these cells do not develop memory against pathogens, they cannot be at the basis of vaccination strategies which aim at the induction of immunological memory against these pathogens.

4. Live Attenuated Vaccines

The use of live attenuated influenza vaccines (LAIVs) is of interest since it results in viral protein synthesis in infected antigen-presenting cells which is a prerequisite for the efficient induction of virus-specific CTL responses [91–94]. LAIVs also induce antibody responses and, thus, have the capacity to induce both virus-specific CTL and B cells which both contribute to protective immunity. They are currently used in the United States and in Russia, and request of approval for their use in Europe has been submitted.

LAIVs have been obtained by adaptation to replicate at low temperatures (25–33°C). The use of these cold-adapted viruses results in mild infections of the upper respiratory tract only. It has been shown in humans that LAIVs induce stronger virus-specific T-cell responses than inactivated vaccines [94, 95]. Intranasal administration of H3N2 LAIV-afforded mice partial protection against infection with H1N1 virus. LAIV-vaccinated mice that were depleted of CD8⁺ T cells were not protected and did not survive H1N1 challenge infection [96]. Furthermore, seasonal LAIV induced strong CTL response in mice and afforded protection against 2009 pH1N1 virus whereas an inactivated vaccine did not [97]. Similar findings were observed by others, and the contribution of T cells in protection against 2009 pH1N1 was confirmed after depletion of these cells [98].

LAIVs based on nonpathogenic H5N2 viruses also provided protection against challenge with highly pathogenic

H5N1 in mice, which correlated with the induction of cross-reactive antibodies but also with cross-reactive T cells [99].

Another strategy to attenuate influenza viruses is to delete part of the nonstructural protein 1 (NS1) [100–102]. This protein is known to be an antagonist of IFN α . Truncation of NS1 renders this protein nonfunctional causing attenuation of the virus [102]. It has been shown in mice that influenza virus, with altered NS1 genes induce potent and protective memory T-cell responses [103].

Also a live attenuated M1 mutant H1N1 virus with an attenuated phenotype *in vivo* was generated [104]. This live attenuated mutant virus also induced broadly cross-reactive immunity against H3N2 and H5N1 viruses, which was shown to be based on both humoral and cellular responses by adoptive transfer experiments.

5. DNA Vaccines

DNA vaccines have the advantage that they can be produced rapidly and at low cost. DNA vaccines encode for one or several proteins of influenza viruses and induce an immune response targeting the encoded protein [105].

Typically, plasmids are constructed with the gene of interest, for example, the NP gene, under control of a strong eukaryotic promoter, for example, the CMV promoter. Upon immunization of the plasmid by injection, electroporation, or gene gun delivery, the gene is expressed in cells that have taken up the plasmid (e.g., myocytes or dendritic cells). Then, the proteins are synthesized in the cytosol of these cells. After processing of these proteins, immunogenic peptides will be generated and presented by MHC class I molecules to virus-specific T cells [106].

The design of DNA vaccine is complex. Over the years, it has been shown that numerous factors play a role in the efficiency of expression such as the promoter, the G/C content (sequences rich in C/G are likely to form secondary structure that inhibit translation), supercoiling that increase transfection efficiency, polyadenylation that enhance stability of mRNA, and codon optimization (for review see [107]).

It has been shown in mice that the administration of DNA vaccine encoding the NP protein of influenza induced a strong CTL response which correlated with protection against challenge infection with homologous or heterologous virus [108]. Numerous studies have confirmed these results with DNA vaccines expressing NP, M1, or HA proteins in various animal models [109–114]. One study evaluated the delivery of the vaccine by *in vivo* electroporation instead of the classical epidermal route. They showed that, in mice, ferrets, and nonhuman primates, this route of delivery induce protective humoral and cellular immunity [115].

Recently, a phase I clinical trial was performed with a candidate influenza DNA vaccine. The vaxfectin-adjuvanted plasmid DNA vaccines encoding influenza H5 HA, NP, and M2 were able to elicit T-cell responses against HA in most of the subjects and against NP and M2 in some of them [116].

Safety remains a concern for DNA vaccination. There might be a risk of integration into the host genome, which may increase the risk of malignancies or tolerance induction [117].

6. Vectored Vaccines

Various viruses can be used as viral vectors to deliver foreign antigens. As for LAIV, the use of viral vectors caused infection of cells, which would allow endogenous antigen processing and MHC class I restricted presentation. Several viruses have been considered as potential vector vaccine candidates and were able to induce CTL response such as baculovirus [118], vesicular stomatitis virus [119, 120], and Semliki Forest virus [121]. Adenovirus and poxviruses, like modified vaccinia virus Ankara (MVA), have been studied extensively for the delivery of influenza antigens. The design and production of such vaccines have been reviewed elsewhere [122–124].

Recombinant adenoviruses that are unable to replicate in human cells and that encode one or more genes of interest such as the HA, NP, and M1 genes, can be produced. Using such recombinant, viruses protective T-cell responses were induced in mice [21, 125–132] and chickens [132, 133]. Recently, it was also demonstrated that an adenovirus-based vaccine expressing HA, NP, and M1 of the 2009 pH1N1 virus induced protective humoral and cellular immunity against homologous challenge and partial protection against challenge with a heterologous virus [134].

MVA-based influenza vaccines have been studied in various animal models extensively [124, 135–137]. These vector vaccines conferred protection and induced virus-specific CTL responses [138–140].

Recently, MVA vectors encoding the NP and M1 genes were evaluated in a phase I clinical trial and were shown to be safe and immunogenic. These candidate vaccines also induced virus-specific CD8⁺ T-cell responses more efficiently compared to other vaccination strategies [141].

7. Other Vaccines and Adjuvants

In addition to the vaccine formulation described above, other vaccine formulations have been described able to induce virus-specific CD8⁺ T-cell responses. For example, virus-like particles, which can be produced after expressing influenza virus antigens in (insect) cells [142], have been shown to induce CTL responses in mice [143, 144] and in chickens [145]. Also, with gamma-irradiated influenza A virus preparations, protective immunity was induced in mice against infection with homologous and heterologous influenza A viruses. Adoptive transfer experiments showed that protective immunity was mediated by virus-specific T cells [146].

Specific adjuvant systems like immune-stimulating complexes (ISCOMs) can be used for the induction of virus-specific CTL responses. ISCOMs consist of cholesterol, phospholipids, viral proteins, and glycosides of the adjuvant Quil A [147]. In addition to enhancing B-cell responses, the use of ISCOMs also induces strong T-cell responses. Since ISCOMs also facilitate transport of viral protein into the cytoplasm of antigen-presenting cells, it also induces CTL responses [148]. It has been demonstrated in mice that, with ISCOM-based vaccines, heterosubtypic immunity can be

induced, which correlated with the induction of the virus-specific T-cell responses [149, 150]. Also, in humans, virus-specific CTL responses could be induced with ISCOM-based vaccines in addition to antibody responses [151, 152]. For the formulation of virosomes, the membrane glycoproteins of influenza viruses are incorporated into a lipid bilayer containing phospholipids resulting in vesicles of ± 150 nm in diameter [153]. Since the fusion activity of the HA molecules is retained, it would allow delivery of antigens (or plasmid DNA) from the endosomes into the cytosol, allowing the induction of CTL responses [154, 155].

In clinical trials, virosome-based vaccines were more immunogenic in the elderly than conventional vaccines [150, 156–158].

8. Conclusions

There is ample evidence that virus-specific CTLs contribute to protective immunity against influenza virus infections. Because of their cross-reactive nature, virus-specific CTLs afford protection against influenza A viruses of various subtypes.

It should be realized that antibodies, directed against the viral envelope proteins HA and NA, are the primary correlates of protection against infection with influenza A viruses provided that they match the strain causing the infection.

The presence of sufficiently high titers of specific serum antibodies, induced by vaccination or infection, will protect individuals from a subsequent infection. Under these circumstances, the induction or presence of virus-specific CD8⁺ T lymphocytes may be redundant. Therefore, the induction of these antibodies should be the strategy of choice. However, in the case of the emergence of drift variants of seasonal viruses, the available vaccines may not be as efficacious due to a poor antigenic match. In the case of the introduction of a novel pandemic strain, the seasonal vaccines will be poorly protective, and novel pandemic vaccines need to be produced, which is a time-consuming process. Under these circumstances, in which humoral immunity fails to afford protection, the presence of cross-reactive CTL will not prevent infection but will contribute to more rapid clearance of infection and reduce disease severity and mortality. Various vaccination formulations aiming at the induction of virus-specific CTL are currently under development. Future preclinical and clinical testing need to provide information on the effectiveness of these vaccines. In a pandemic scenario, vaccines that induce cross-protective CTL could be used for emergency vaccination until vaccines become available that induce antibodies of the proper specificity. Especially, immunogenically naïve subjects, like young children, that have not previously experienced influenza virus infection may benefit from such a strategy.

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