Chapter 5

The effect of pre-existing immunity on the capacity of influenza virosomes to induce cytotoxic T lymphocyte activity

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Abstract

Virosomes are reconstituted viral envelopes that retain the receptor-binding and membrane fusion capacity of the virus they are derived from. Antigens encapsulated in virosomes can be delivered into the MHC class I route of antigen presentation, which may result in induction of cytotoxic T lymphocyte (CTL) responses. However, virosomes derived from, for example, influenza virus would potentially be subject to pre-existing immunity directed against influenza virus components, which might hamper their ability to induce a CTL response. In this study, CTL induction in mice by viroome-encapsulated ovalbumine (OVA) protein was investigated in the absence or presence of influenza virus-specific immunity. It was discovered that CTL induction by virosomes was only slightly reduced by pre-injection of influenza virus-specific antibodies or pre-exposure to influenza virus. Both pre-treatments resulted in the same level of reduction, suggesting that virus-specific antibodies rather than T cell responses account for the reduction. Furthermore, a booster-immunization greatly enhanced CTL activation, indicating that viroome-specific immunity induced by a prime immunization does not hamper the booster effect. Clearly, CTL induction by virosomes is largely unaffected by influenza virus-specific immune responses.

From these results, it is concluded that CTL induction against a viroome-encapsulated protein antigen is not significantly inhibited by pre-existing humoral or cellular immunity against influenza virus. It is hypothesized that influenza virus-specific antibodies facilitate CTL induction by allowing for Fc-receptor-mediated uptake of opsonized virosomes by professional APC and antigen delivery to the cytosol through viroome-endosome membrane-fusion.
Introduction

Virosomes are reconstituted viral envelopes, which retain the receptor-binding, cell entry, and membrane fusion characteristics of the virus they are derived from. On the other hand, virosomes do not contain the viral genetic material and, therefore, are non-infectious [1]. Virosomes may be produced from a range of enveloped viruses [2-6], however, virosomes derived from influenza virus [7] have been used most often.

The virosomes used in this study are produced by solubilization of influenza virus in detergent, elimination of the viral genome through ultracentrifugation, and subsequent reconstitution of the viral envelope components by selective extraction of the detergent [8]. Compounds can be encapsulated in the lumen of the virosomes or co-reconstituted in the virosomal membranes by addition of these compounds to the solubilized virus envelopes [9]. Virosomes thus created have the major influenza virus glycoprotein, hemagglutinin (HA), protruding from their surface. Through HA, these virosomes bind to sialic acid residues, the cellular receptor for influenza virus, on target cells, are taken up via receptor-mediated endocytosis, and delivered to endosomes [1]. Subsequently, triggered by the mildly acidic pH within the endosomes, HA catalyses a fusion reaction between the virosomal and endosomal membranes [10]. As a result of this fusion reaction, the contents of the virosose is introduced into the cytosol of the cell.

Virosomes have been developed for the in vitro and/or in vivo delivery of proteins, peptides, drugs, and DNA as reviewed in [11-14], as well as for the delivery of siRNA [15]. Cytosolic delivery of protein antigens to professional antigen presenting cells (APC) results in presentation of peptides in the context of major histocompatibility complex (MHC) class I molecules in vitro [16] and induction of cytotoxic T lymphocyte (CTL) responses in vivo [17-19].

Pre-existing influenza virus-specific immunity would generally be expected to be a limitation of the in vivo use of virosomes. As influenza virus is a common virus, many patients that would potentially receive virosomes may have influenza virus-specific antibodies that may neutralize the virosomes before they can achieve their desired effect. On the other hand, influenza virus-specific antibodies have been found to enhance infection of Fc-receptor expressing cells such as professional APC, in a process known as antibody-dependent enhancement (ADE) of infection [20-23]. Via this mechanism virosomes may be targeted to APC and may therefore still induce CTL responses in the presence of influenza virus-specific immunity. The present paper specifically addresses this issue in a murine model system.

Studies on the effect of pre-existing immunity have focused on recombinant viruses demonstrating that virus-specific antibodies or T cell reactivity may substantially reduce the potency
of these vector systems [24]. Neutralization by pre-existing antibodies, specific for the recombinant virus, can prevent infection of target cells [25-27]. Cellular immunity, mainly T cells specific for structural or non-structural proteins of the virus, can eliminate already infected cells [26-31]. Pre-existing immunity, thereby, may reduce transgene expression, which in turn could compromise gene therapy as well as immunotherapy applications of the viral vector system involved. For two delivery systems that are comparable to virosomes, liposomes and virus-like particles (VLP), the effects of pre-existing immunity have been described previously. Neutralizing antibodies directed against antigens or lipids that make up liposomes may result in clearance of liposomes from the bloodstream by phagocytic cells through Fc-receptor-mediated phagocytosis [32-34]. Concerning the effect of pre-existing immunity on VLP, there have been diverse reports. Da Silva et al [35] have shown that the induction of anti-tumor responses by human papillomavirus (HPV) VLP is inhibited by transfer of HPV-specific antibodies that block receptor binding. Liu et al [36], on the other hand, did not observe an effect of HPV-specific antibodies on the induction IFNγ-producing CD8+ T lymphocytes by HPV-derived VLP. Instead, they found that the induction of CTL responses by HPV-derived VLP was solely hampered by HPV-specific cellular immunity. VLP derived from hepatitis B virus were not found to be hampered by hepatitis B virus-specific immunity [37].

In order to investigate the effect of influenza virus-specific immunity on the capacity of influenza virosomes to induce a CTL response against an encapsulated antigen, we have, in the current study, used virosomes containing the model protein antigen ovalbumin (OVA) to induce OVA-specific CTL responses in mice pre-exposed to influenza virus.

**Materials and methods**

**Cells**

EL-4 and EG7OVA are C57BL/6 mouse lymphoma cell lines (H-2b). EG7OVA is a stable transfectant of EL-4, expressing the cDNA of chicken OVA [38]. Both cell lines were cultured in IMDM+Glutamax medium (Invitrogen, Paisley, UK) supplemented with 10% FCS (Bodinco B.V., Alkmaar, The Netherlands), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen).
Mice

Specified pathogen-free female C57BL/6 mice were purchased from Harlan CPB (Zeist, The Netherlands) and used at 8 to 10 weeks of age. They were kept according to institute guidelines. All animal experiments were approved by the local Animal Experimentation Ethical Committee (DEC UMCG Groningen).

Virus

Purified influenza virus of the H3N2 subtype (A/Panama/2007/99) was kindly provided by Solvay Pharmaceuticals (Weesp, The Netherlands). For the pretreatment of mice with influenza virus, 500 hemagglutination units (HAU) of virus were diluted in 200 µl HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA) and injected intraperitoneally (i.p.)

Virosomes

Virosomes were prepared as described previously [1;8]. In short, influenza virus (1.5 µmol of viral membrane phospholipid) was solubilized in 700 µl HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA) containing 100 mM octa(ethyleneglycol)-n-dodecyl monoether (C_{12}E_{8}) (Calbiochem, San Diego, CA, USA). After an overnight incubation at 0°C, the RNA containing nucleocapsids were removed by ultracentrifugation. 3 mg/ml OVA (grade VII; Sigma, St. Louis, MO, USA) was added to the supernatant containing the phospholipids and glycoproteins of the virus in C_{12}E_{8}. Subsequently, OVA-containing virosomes were formed by extraction of the detergent C_{12}E_{8} with BioBeads SM2 (Bio-Rad, Hercules, CA, USA). The virosomes were separated from non-encapsulated OVA on a discontinuous sucrose density gradient. Finally, the virosomes were dialyzed against HNE buffer. Empty virosomes were prepared by not adding OVA. Pyrenelabeled virosomes were prepared by adding 10 mol% 1-hexadecanoyl-2-(1-pyrenedecanoyl)-syn-glycero-3-phosphocholine (pyrene PC) (Molecular Probes, Leiden, The Netherlands) relative to viral phospholipids to the supernatant prior to reconstitution. Virosomal phospholipid content was determined by phosphate analysis [39]. Virosomal protein (mainly HA) was determined according to Lowry [40]. The amount of OVA in the virosm membrane preparations was determined by encapsulating FITC-labeled OVA (Molecular Probes) and comparing the fluorescence intensity in the virosm sample with that of a reference series of OVA-FITC. For immunization experiments, the equivalent of 50 nmol phospholipids of OVA-virosomes (i.e. ~2.5 µg of OVA protein) in 200 µl HNE was injected i.p.
Influenza virus-specific immune serum

Influenza virus-specific immune serum was obtained from mice that had been vaccinated i.p. twice with 50 nmol of empty virosomes in Alhydrogel Adjuvant (40%) (GERBU Biotechnik, Gaiberg, Germany) in a total volume of 200 µl. Three weeks after the second vaccination, mice were bled and the blood was allowed to coagulate overnight at 4°C. Serum was acquired after centrifugation. The serum had an IgG titer of $10^5$ and a hemagglutination inhibition (HAI) titer of $8 \times 10^3$ against influenza virosomes. For transfer studies, 200 µl immune serum was injected intravenously (i.v.) via the orbita plexus into naïve recipients.

ELISA

ELISA plates with high binding capacity (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with 200 ng influenza virus A/Panama/2007/99 subunit vaccine (Solvay Pharmaceuticals, Weesp, The Netherlands) in 50 µl coating buffer (0.05 M carbonate-bicarbonate pH 9.6) per well at 37°C. Plates were washed once with coating buffer and blocked with 2.5% milk powder solution in coating buffer for 45 min at 37°C. After the blocking step, plates were washed with coating buffer and twice with PBS-Tween (PBS + 0.02% Tween 20). Serum samples were applied to the plates and sequentially diluted. After a 1.5-hr incubation at 37°C, plates were washed 3 times with PBS-Tween and incubated for 1 hr at 37°C with horseradish peroxidase conjugated anti-mouse IgG (SouthernBiotech, Birmingham, AL, USA). Plates were washed with PBS-Tween and PBS and the staining reaction was started by applying 0.02% o-phenylene diamine dihydrochloride in 100 mM phosphate buffer pH 5.6 complemented with $\text{H}_2\text{O}_2$. The reaction was stopped after 30 min by adding 2 M $\text{H}_2\text{SO}_4$. Absorbance was measured at 492 nm. The dilution at which absorbance was 0.2 above background was used to deduce the antibody titer.

IFNγ ELISPOT assay

ELISPOT analysis was done essentially according to a method previously described [41]. ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with purified anti-mouse IFNγ (rat IgG1) (BD Pharmingen, San Diego, CA, USA) at 37°C. Sterile PBS-Tween (PBS + 0.02% Tween 20) was used to wash the plates three times. Next, the plates were incubated with blocking buffer (PBS containing 4% RIA Grade BSA (Sigma)) for 1 hr. Freshly isolated spleen cells, obtained 10 days after the mice received their last immunization, were plated into wells and serially diluted in medium containing 5% FCS. The splenocytes
were incubated overnight with or without 100 ng/ml of the OVA peptide 257–264 (SIINFEKL, produced by H. Hilkmann, The Netherlands Cancer Institute, Amsterdam, The Netherlands). After overnight incubation, cells were lysed by a 10-min exposure to water and plates were washed five times with PBS-Tween. Plates were, subsequently, incubated for 1 hr with biotinylated anti-mouse IFN-γ mAb (BD Pharmingen), followed by a 1-hr incubation with streptavidin-alkaline phosphatase (BD Pharmingen). Spots were developed by adding the substrate 1 mg/ml 5-bromo-4-chloro-3-indolylphosphate in water containing 6 mg/ml agarose (Sigma), 9.2 mg/ml 2-amino-2-methyl-1-propanol (Sigma) and 0.08 μl/ml Triton X-405 and incubation at 37°C for 2 hr. After an additional overnight incubation at 4°C, spots per well were counted in triplicate and the number of spots in wells containing equal numbers of unstimulated cells was subtracted from the numbers counted in wells containing stimulated cells.

**CTL assay**

Ten days after the last immunization, freshly isolated spleen cells were co-cultured with irradiated (100 Gy) EG7OVA cells at an effector-to-stimulator ratio of 25:1 in 25 cm² culture flasks, placed upright. After 5 days of culture, 4 U/ml of recombinant human IL-2 (Strathmann Biotech, Hamburg, Germany) was added to the target cells. A standard 4-hr ⁵¹Cr release assay in triplicate determinations was performed after two additional days of culture. EG7OVA cells or SIINFEKL-loaded EL-4 cells were used as targets. EL-4 cells without peptide were used as negative control target cells. Target cells were labelled for 1 hr with 3.7 MBq ⁵¹Cr/10⁶ cells in 50 μl medium (⁵¹Cr was from MP Biomedicals, Asse-Relegem, Belgium). During the ⁵¹Cr-labeling period, SIINFEKL was added at a final concentration of 30 μg/ml to obtain SIINFEKL-loaded EL-4 cells. Specific lysis was calculated according to the following formula: % specific lysis = (experimental release − spontaneous release) / (maximal release − spontaneous release) x 100. Spontaneous release was determined from target cells incubated without effector cells and maximal release was determined from target cells incubated with medium containing 0.5% Triton X-100. The spontaneous ⁵¹Cr release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

**Fusion assay**

Virosome fusion with erythrocyte ghosts, as a measure for virosome-receptor binding, was determined using a lipid mixing assay based on pyrene excimer fluorescence [9]. Fusion at pH 5.5 was continuously monitored at 37°C by the decrease of pyrene excimer fluorescence.
in an AB2 fluorometer (SLM/Aminco, Urbana, IL, USA). The extent of fusion was calculated based on the decrease in pyrene excimer fluorescence at 480 nm upon acidification by the addition of 0.1 M morpholinoethanesulfonic acid (MES), 0.1 M acetic acid to achieve pH 5.5. The excimer fluorescence of unfused virosomes was used as the reference value for 0% fusion. The fluorescence after addition of \( C_{12}E_8 \), added to achieve infinite dilution of the pyrene PC, as the 100% fusion level.

Results

Induction of CTL activity in mice after transfer of influenza virus-specific serum

To investigate whether influenza virus-specific immunity affects CTL induction by virosomes, we compared CTL priming in virus-immune mice with that in naïve mice. For most viral vectors which are hampered by vector-specific immunity, vector-neutralizing antibodies are believed to represent the primary cause for the reduction in efficacy [24-27]. Therefore, the effect of neutralizing antibodies on CTL induction by influenza virosomes was investigated first. High influenza virus-specific antibody titers were generated by i.v. transfer of 200 µl immune serum to naïve mice. The mice were subsequently immunized with 2.5 μg of virosome-encapsulated OVA 6 hr after serum transfer. Two weeks later the mice received a second injection of serum and were boosted with OVA-virosomes 6 hr afterwards. Specific IgG titers, induced by serum transfer were determined by ELISA at the time of immunization with OVA-virosomes. Serum transfer resulted in reproducibly high influenza virus-specific antibody titers of approximately $10^4$, corresponding with the approximate 10-fold dilution of the transferred serum (data not shown). This 10-fold dilution would correspond with an HAI titer of $10^2$. In man, an HAI titer ≥40 is considered protective against influenza virus infection [42].

Ten days after the final immunization, mice were sacrificed and spleens were collected. The frequency of IFNγ-producing CTLs in the freshly isolated splenocytes was determined by ELISPOT. Immunization with OVA-virosomes resulted in 120-230 IFNγ-secreting cells per $10^6$ splenocytes in mice that were immunologically naïve for influenza virus. In mice that had received serum transfer, immunization with OVA-virosomes resulted in 50-130 IFNγ-secreting cells per $10^6$ splenocytes (Figure 1A). Cytolytic activity of the splenocytes was determined with a $^{51}$Cr release assay after 7 days of in vitro restimulation. Spleen cells from naïve mice or from mice that received immune serum before immunization with OVA-virosomes both showed high OVA-specific cytolytic activity. At an effector cell to target cell (E:T) ratio of 30 to 1, 59% spe-
specific lysis was detected in control mice that were not pretreated with immune serum, whereas spleen cells from pretreated mice displayed 41% specific lysis (Figure 1B).

Taken together, these results indicate that influenza virosomes do induce a CTL response against an encapsulated antigen despite the presence of high levels of influenza virus-specific circulating antibodies.

**Figure 1: CTL induction in mice with influenza virus-specific antibodies.**

Mice were pre-injected with 200 µl anti-influenza serum with an IgG titer of 10^5 (open squares, n=4) or buffer (black triangles, n=4) and immunized i.p. with 50 nmol OVA-virosomes 6 hr later. Fourteen days later, mice were again injected with anti-influenza serum or buffer and booster immunized. Control mice that received two buffer injections were also included (black squares, n=2). Ten days after the last injection, mice were sacrificed and spleens were isolated. **A.** Freshly isolated splenocytes were incubated overnight with or without 100 ng/ml of the OVA peptide SIINFEKL. The numbers of IFNγ-producing cells were determined by subtracting the number of spots obtained without peptide stimulation from the number of spots obtained with peptide stimulation. IFNγ-production was determined in a triplicate well assay. The mean numbers of spots per group are shown with error bars representing standard error of the mean. **B.** Cytolytic activity against SIINFEKL-loaded EL-4 cells was determined in a triplicate well assay after a 7-day *in vitro* restimulation. The mean levels of specific lysis at different effector cell to target cell ratios are shown with error bars representing standard error of the mean.
**Induction of CTL activity in mice pre-exposed to influenza virus**

Because influenza virus-specific antibodies did not severely affect CTL priming, we set out to investigate whether a cellular response against influenza virus has an effect on the capacity of virosomes to induce CTL activity against an encapsulated antigen by pre-exposure of the mice to live influenza virus. Besides inducing antibody titers, infection with influenza virus results in a cellular immune response against the virus [43;44]. Mice were exposed i.p. to 500 HAU influenza virus and immunized with 2.5 μg of OVA, encapsulated in virosomes 14 days later. A booster immunization was given an additional 14 days later. Exposure to influenza virus resulted in a specific IgG antibody response at the time of immunization with OVA-virosomes. The titers were of the same magnitude as the titers generated in the serum transfer experiment presented above (data not shown). Ten days after the booster immunization, mice were sacrificed and spleens were collected to determine the frequency of IFNγ-producing CTLs. Immunization with OVA-virosomes resulted in approximately 170 IFNγ-secreting cells per 10^6 splenocytes in naïve mice as well as in mice that were exposed to influenza virus (Figure 2A).

Spleen cells from naïve mice as well as spleen cells from mice that were exposed to influenza virus before immunization with OVA-virosomes showed high OVA-specific cytotoxicity after *in vitro* restimulation. At an E:T ratio of 30 to 1, naïve mice displayed approximately 52% and mice pre-exposed to influenza virus displayed 41% specific lysis (Figure 2B). This experiment indicates that the immune responses induced by pre-exposure to influenza virus, including a cellular response, do not prevent subsequent CTL induction by OVA-virosomes.

**The effect of a booster-immunization**

Even when a vector or carrier system is not subject to pre-existing immunity, vector- or carrier-specific immunity induced by a prime immunization may hamper homologous prime-boost immunization protocols. To determine whether a booster immunization with virosomes is hampered by prime-induced virosome-specific immunity, we investigated how much a booster immunization with virosomes contributes to CTL activation in our immunization protocol. In Figure 3, we compared the effect of a single immunization of OVA-virosomes with two immunizations. A prime-immunization with OVA-virosomes resulted in an influenza virus-specific antibody titer of 9x10^3 at the time of the booster-immunization (data not shown). Despite this high antibody titer, a homologous prime-boost immunization with OVA-virosomes resulted in about 57% OVA-specific cytolysis at an E:T ratio of 30 to 1, while a single immunization resulted in approximately 15%. This experiment shows that a virosome booster immunization...
is not only essential to enhance CTL activity, but is also effective in the presence of influenza virus-specific immunity.

**The effect of influenza virus-specific antibodies in vitro**

The above experiments show that in mice pre-exposed to influenza virus or treated with immune serum, CTL induction by influenza virus-derived virosomes is only marginally reduced compared to CTL induction in naïve control mice. This is remarkable as neutralization by
Figure 3: The effect of a booster immunization.

A. Mice received a single injection i.p. with 50 nmol OVA-virosomes (black triangles, n=2) or buffer (black squares, n=1) and were sacrificed 24 days later. B. Other mice received 2 injections with 50 nmol OVA-virosomes (black triangles, n=4) or buffer (black squares, n=1) 14 days apart and were sacrificed 10 days after the last injection. Isolated spleen cells were restimulated in vitro for 7 days and subsequently cytolytic activity against SIINFEKL-loaded EL-4 cells was determined in a triplicate well assay. The mean levels of specific cytolysis at different effector cell to target cell ratios are shown with error bars representing standard error of the mean.

pre-existing antibodies has been found to severely abrogate the capacity of many other virus-derived systems to express transgenes or deliver compounds to cells. To investigate to which extent influenza virus-specific antibodies affect the functional characteristics of virosomes, we determined binding of virosomes to the cellular receptor of influenza virus in the presence of anti-influenza virus serum using a membrane fusion assay [45]. Membrane fusion of virosomes with a target membrane requires binding of virosomal HA with its receptor, sialic acid, and a subsequent conformational change of the HA due to a drop in pH. Incubation of virosomes with different aliquots of influenza virus-specific immune serum resulted in a dose-dependent reduction in receptor binding as measured by a decrease in the maximum extent of fusion at pH 5.5 (Figure 4). Incubating 0.7 nmols of virosomes with 3 μl of the serum with an IgG titer of $10^5$ and a HAI titer of $8\times10^3$ resulted in a total block of HA-receptor binding, as visualized by a total absence of fusion activity. This experiment demonstrates that the virus-specific antiserum used in this study does neutralize receptor binding of the virosomes.
Discussion

Here we have investigated the effect of (pre-existing) influenza virus-specific immunity on the capacity of influenza virosomes to act as an antigen delivery system for CTL induction. The presented experiments demonstrate that virosome-mediated CTL induction in mice is only marginally affected by prior transfer of influenza virus-specific immune serum to the mice or pre-exposure of the animals to live influenza virus. We also found that a booster-immunization strongly amplifies a CTL response, despite the fact that virosome-specific immunity is induced by the prime immunization. Furthermore, it was found that neutralizing antibodies do completely block binding of virosomes to their cellular sialic acid receptor.

Figure 4: The effect of influenza virus-specific antibodies in vitro.
Fusion of virosomes with a target membrane in the presence of influenza virus-specific immune serum was determined as a measure for binding of virosomal HA with its receptor. Virosomes were incubated with increasing amounts of serum with an IgG titer of $10^5$ for 20 min, subsequently mixed with erythrocyte ghosts and fusion was initiated by acidification to pH 5.5.
The small reduction in CTL induction by influenza virus-specific immunity appears to be primarily due to humoral immunity. Pre-injection of immune serum and pre-exposure to influenza virus resulted in a similar level of CTL induction by virosomes. Yet, pre-exposure to influenza virus induces both virus-specific antibodies and cellular responses [43;44]. As there was no additional reduction in cytolysis, CTL induction by virosomes is apparently not hindered by influenza virus-specific cellular immunity.

Comparison of a single immunization protocol with a homologous prime-boost protocol shows that a booster immunization is essential for the induction of a strong CTL response and is not hindered by prime-induced virosome-specific responses. The first experiments of this study showed that CTL induction by virosomes is not hindered by influenza virus-specific immunity. Yet, one could argue that the virosome-specific immune response induced by a prime immunization with OVA-virosomes differs from an influenza virus-specific response generated by serum transfer or pre-exposure to influenza virus. For example, we have shown that, in OVA-virosomes, up to 40% of the encapsulated OVA protein is associated with the virosomal membrane, possibly also at the external surface of the virosomes [18]. We have furthermore demonstrated that an immunization with virosomes induces antibody responses against the encapsulated protein antigen [19]. Therefore, a booster immunization is subject to OVA-virosome-specific immunity, which may include OVA-specific antibodies. These OVA-specific antibodies would not neutralize virosomes by blocking receptor binding, but may facilitate complement binding and might thereby hinder CTL induction. However, this is clearly not the case as CTL activation by a booster immunization with OVA-virosomes is not abrogated by prime-induced immunity.

The exact mechanism by which virosomes evade neutralization by vector-specific immunity remains unclear. Based on our findings that CTL induction is hardly hampered by pre-existing immunity but that binding of virosomal HA with its sialic acid receptor is blocked \textit{in vitro} by influenza virus-specific serum, we hypothesize that virus-specific antibodies might facilitate CTL induction via a different route of antigen delivery. Opsonized virosomes may be targeted to Fc-receptor-expressing cells such as professional APC and might subsequently be taken up through Fc-receptor-mediated endocytosis. After cellular uptake, the low pH within endosomes may then still enable membrane-fusion as influenza-neutralizing antibodies are not directed against the membrane fusion-catalyzing HA2 subunit of HA but against epitopes on the sialic acid receptor-binding subunit HA1 at the membrane-distal tip of the molecule [10]. Furthermore, the drop in pH may cause detachment of these antibodies thereby allowing the virosomal HA to induce membrane fusion and subsequent antigen delivery to the cytosol. Accordingly, it has been described previously that opsonized influenza virus can infect cells via
Fc-receptor-mediated uptake in a process of ADE [20-22;46].

In addition, opsonization may also facilitate CTL activation by other mechanisms. Opsonization could enhance targeting of virosomes to lymphoid organs and may thereby facilitate more efficient antigen transfer to APC. Opsonizing antibodies can also modulate immune responses via Fc-receptor-independent mechanisms [47]. Binding of complement factors C3b or C1q to opsonizing antibodies has been described to facilitate ADE via complement receptors [21;48] and this may also play a role in the uptake of antigen from virosomes opsonized by antibodies. Furthermore, Simitsek et al [49] have shown that attached antibodies may enable more effective intracellular processing of the opsonized antigen, thereby allowing for enhanced loading of peptides on MHC molecules and improved CTL induction.

In conclusion, this study shows that influenza virosomes efficiently deliver proteins for antigen processing and class I MHC-restricted CTL priming, even in the presence of influenza virus-specific immunity.

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