Protective immunity against influenza in HLA-A2 transgenic mice by modified vaccinia virus Ankara vectored vaccines containing internal influenza proteins

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ABSTRACT

Background: The emergence of novel strains of influenza A viruses with hemagglutinins (HAs) that are antigenically distinct from those circulating in humans, and thus have pandemic potential, pose concerns and call for the development of more broadly protective influenza vaccines. In the present study, modified vaccinia virus Ankara (MVA) encoding internal influenza antigens were evaluated for their immunogenicity and ability to protect HLA-A2.1 transgenic (AAD) mice from infection with influenza viruses.

Methods: MVAs expressing NP (MVA-NP), M1 (MVA-M1) or polymerase PB1 (MVA-PB1) of A/California/4/09 (CA/09) virus were generated and used to immunize AAD mice. Antibodies and CD8+T cell responses were assessed by ELISA and ELISPOT, respectively, and challenge experiments were performed by infecting vaccinated mice with CA/09 virus.

Results: CD8+T cells specific to immunodominant and subdominant epitopes on the internal influenza proteins were elicited by MVA-based vectors in AAD mice, whereas influenza-specific antibodies were detected only in MVA-NP-immunized mice. Both M1- and NP-based MVA vaccines, regardless of whether they were applied individually or in combination, conferred protection against lethal influenza virus challenge.

Conclusion: Our data further emphasize the promising potential of MVA vector expressing internal antigens toward the development of a universal influenza vaccine.

Introduction

Influenza A viruses are widely distributed in nature and can infect both animals and humans [1]. Occasionally, zoonotic spillover events may lead to the emergence of novel strains of influenza A viruses with hemagglutinins (HAs) that are antigenically distinct from those circulating in humans, and thus have pandemic potential [1,2]. Monovalent candidate vaccines that elicit HA-specific neutralizing antibodies have been produced against potentially pandemic strains [3]. Nevertheless, the unpredictable variability of influenza A viruses poses concerns and calls for the development of more broadly protective influenza vaccines [4].

Although neutralizing antibodies to the globular HA are most effective for the prevention of illness, new and innovative strategies aimed at inducing cross-reactive antibodies that bind to conserved portions of the HA stalk show promise for development of a universal influenza vaccine [5–8]. Furthermore, the induction or recall of CD8+T cells against conserved epitopes of the internal viral proteins, which are very poorly elicited by currently inactivated influenza vaccines, would effectively contribute to reducing viral load and limit disease severity after infection with a heterosubtypic influenza virus [9–13]. In particular, the rapid boosting of these cross-reactive influenza-specific memory T cells, which accumulate in the lungs and lymphoid tissues in humans after recovery from influenza virus infections, could be extremely beneficial to provide protection against severe influenza virus infections [14–16].

Several studies have been performed to determine the efficacy of viral vector vaccines that were engineered to express the conserved antigens of the influenza virus [17–19]. In particular, replication-deficient modified vaccinia virus Ankara (MVA) constructs encoding internal influenza proteins have been demonstrated to be safe and highly immunogenic in both animal models and human volunteers [20–23]. In this context, the co-administration of MVA, expressing a fusion protein of influenza A nucleoprotein (NP) and matrix protein (M1) (MVA-NP+M1), with seasonal vaccine simultaneously has been shown to boost T cell responses and increase some influenza-specific antibodies compared with a group
Peptides

Three peptide epitopes for mouse MHC class I molecules, H2-D\(^b\)-NP\(_{366}\), H2-K\(^b\)-PB1\(_{703}\) and H2-K\(^b\)-M1\(_{128}\), and ten peptides that bind the HLA-A2.1 molecule and are conserved among diverse influenza subtypes were synthesized by Primm (Italy). Peptide purity was > 90% in all cases, and the identity of the peptides was verified by using spectrometry. The peptides were dissolved in DMSO at 10 mg/ml and stored at −20 °C.

IFN-γ ELISPOT assays

Spleens of mice (6–7 mice per group) were collected seven days after immunization or virus infection and immediately assayed for antigen-specific IFN-γ-producing cells using an IFN-γ ELISPOT assay. Single-cell suspensions from the lymphocytic populations were cultured with the indicated synthetic peptides or DMSO on anti-IFN-γ-coated plates at 37 °C for 36–40 h. Colored spots representing IFN-γ-releasing cells were reported as the number of spot-forming cells (SFC) per 10\(^6\) spleen cells.

Antibody analysis

Serum samples were collected from mice vaccinated with MVA viruses immediately before virus challenge and tested for the presence of influenza-specific antibodies in ELISA using detergent–disrupted CA/09 virus [27].

Results

Generation and analysis of recombinant MVA viruses

Western blot was performed by using polyclonal specific antisera to determine the transgene expression by MVA recombinant viruses [26].

Vaccination of AAD mice and virus challenge

AAD mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and animal experiments were performed at the Istituto Superiore di Sanità in compliance with institutional guidelines and approved protocols [25]. Groups of mice (6–8 weeks) were vaccinated intramuscularly (i.m.) twice, three weeks apart, with 10\(^7\) pfu of the MVA-M1, MVA-NP or MVA-PB1 viruses, given as single or a three-MVA virus combination in separate injection sites. Four weeks after the last immunization, mice were anesthetized with Avertin and challenged intranasally (i.n.) with 2.50% lethal doses (LD\(_{50}\)) of CA/09 virus in 45 μl volume. Control mice received MVA-wt virus or an equal volume of PBS. Mice were then monitored for survival and weight loss for 14 days after infection.
and specificity of primary CD8+T cell responses that were elicited against the internal proteins of influenza virus following immunization of the AAD mice with single recombinant MVA viruses. To this end, a panel of HLA-A2-restricted influenza derived CD8+T cell epitope peptides (see Table 1) consisting of 3 NP epitopes, 4 PB1 epitopes and 3 M1 epitopes was used in ex vivo IFN-γ ELISPOT assays because these epitopes are highly conserved among different influenza subtypes [29–31]. Moreover, the murine peptide epitopes, H2-Db-NP366, H2-Kb-PB1703, and H2-Kb-M1128, were also used in the assay to assess the specific H2-restricted-CD8+T cell responses [32−34].

Mice immunization with either MVA-M1 or MVA-NP virus elicited CD8+T cell responses that were mainly detectable for the immunodominant epitopes A2-M158 and H2-Db-NP366, respectively. Reproducible T cell responses were also observed for the overlapping peptide A2-M159 in the MVA-M1-immunized mice, whereas lower numbers of responder T cells were consistently measured for the other subdominant epitopes (Figure 2).

Interestingly, vaccination of mice with MVA-PB1 virus elicited detectable responses specific to peptide epitopes A2-PB1501 and H2-Kb-PB1703, whereas no significant T cell frequencies were revealed against the other subdominant epitopes of PB1 protein. When mice were vaccinated with the three-virus combination, CD8+T cell responses to the specific peptides were similar to those measured in mice vaccinated with the single MVA viruses (Figure 2). Infection of AAD mice with sublethal doses (0.1 LD50) of CA/09 virus generated A2-M158-specific and H2-D-Db-NP366-specific CD8+T cells with similar frequency. In addition, significant numbers of responding T cells to at least six subdominant epitopes of those listed in Table 1 were measured in these mice. Among them, CD8+T cells specific to the peptide A2-M159, overlapping the immunodominant A2-M158, were prevalent, followed by those specific to H2-Kb-PB1703, A2-PB1501, H2-Kb-M1128, A2-M1130 and A2-NP329. Thus, a similar pattern in reactivity was detected in mice following MVA-based immunization or influenza virus respiratory infection.

To investigate whether the MVA-based vaccines generated influenza A virus-specific antibodies, sera from vaccinated mice were tested in ELISA against detergent-disrupted CA/09 virus. The results showed that MVA-NP vaccination, as a single virus and in combination with the other MVAs, induced robust serum IgG titers, whereas no detectable IgG responses were induced by the PB1- and M1-based vaccines (Figure 3).

Together, these data indicate that vaccination with MVA-based vectors expressing the internally conserved influenza proteins elicits CD8+T cell responses against immunodominant and subdominant epitopes and antibodies against NP protein, regardless of whether they were applied alone or in combination.

**Protective efficacy against a lethal influenza virus challenge**

To determine whether MVA-based vaccines could elicit protective immunity, AAD mice were immunized and then challenged with 2 LD50 of the CA/09 virus. All mice

Table 1. Influenza A virus-derived MHC class I restricted T cell epitopes included in the study.

| Peptides | Sequence | Position | MHC restriction |
|----------|----------|----------|-----------------|
| M1-58    | GLGIFVFgL | 58–66    | HLA-A2.1        |
| M1-59    | ILGFYFTLV | 59–68    | HLA-A2.1        |
| M1-128   | MGLYINRM  | 128–135  | H2-Kb           |
| M1-130   | GLYNRMGA  | 130–138  | HLA-A2.1        |
| PB1-407  | MNGMGMFML | 407–415  | HLA-A2.1        |
| PB1-413  | NMLSTVLG  | 413–421  | HLA-A2.1        |
| PB1-501  | FVANFSMEL | 501–509  | HLA-A2.1        |
| PB1-505  | FSMELPSFGV | 505–514  | HLA-A2.1        |
| PB1-703  | SSSRRPPVGG | 703–711  | H2-Kp           |
| NP-275   | CLPACVCYG | 275–283  | HLA-A2.1        |
| NP-329   | QLVWMACHSA | 329–339  | HLA-A2.1        |
| NP-458   | FQGRGFGEL | 458–466  | HLA-A2.1        |
| NP-366   | ASNENWEYT | 366–374  | H2-D-Db         |

Note: Murine MHC haplotypes are indicated in bold.
virus combination showed 16% weight loss and 100% survival rate (Figure 4).

To assess the epitope specificity of CD8+ T cells after viral challenge, mice of each group were sacrificed on day 7 p.i., and IFN-γ production was measured by ELISPOT assay from splenocytes in the presence of the selected peptides. High levels of CD8+ T cells specific to A2-M158 and H2-Db-NP366 were found in mice previously vaccinated with the three-MVA virus combination that correlated with the survival rate of these mice (Figure 5). Notably, the frequencies of immunodominant epitopes A2-M158 and H2-Db-NP366 increased at least five-fold in the spleens after viral challenge, whereas the frequencies of the subdominant epitopes were similar to those observed in unvaccinated mice. Similar results were obtained from mice immunized with MVA-M1 or MVA-NP virus (data not shown).

Overall, our results show that protective cellular immune responses can be induced in AAD mice following systemic immunization with MVA-based vectors expressing the conserved internal antigens M1 and NP.

Discussion

In the present study, we evaluated the CD8+T cell responses to internal viral proteins elicited by MVA-based vectors in AAD mice. We found that CD8+T cells specific to immunodominant and subdominant epitopes could be detected in these mice. Importantly, such CD8+T cell-mediated immunity appears to be associated with
Recombinant MVAs have been widely recognized as suitable vaccine vectors for their safety and immunogenic properties, even in the presence of pre-existing MVA immunity [35,36]. Although MVA vectors expressing various combinations of the internal viral proteins have already been evaluated in other animal models, there is a lack of information on the specificity for both dominant and subdominant epitopes that could be targeted by CD8+ T cells following vaccination. Immune responses to subdominant epitopes may contribute to fight a viral infection, especially in the case of the emergence of cytotoxic T lymphocyte escape variants with mutations in a dominant epitope [37,38]. We recently described that immunization of AAD mice with inactivated virus induced CD8+ T cell responses mainly to the immunodominant epitopes [25]. Here, we investigated whether MVA-delivered antigens could improve the breadth and specificity of primary CD8+ T cell responses that are elicited in AAD mice following vaccination. Our data show that immunization with MVA-M1 virus elicited CD8+ T cells specific to the HLA-A2-restricted immunodominant M158 epitope and, to a lesser extent, the overlapping peptide epitope M159, whereas lower reactivity was measured against the subdominant epitopes A2-M1 130 and H2-Kb-M1128. Mice immunized with MVA-NP virus showed significant serum levels of antibodies against NP protein and high numbers of spleen cells reactive to the immunodominant epitope H2-Db-NP366. Additionally, we could detect the expression of PB1 in cells infected by MVA-PB1 and consistently measure a reactivity to the peptide epitopes A2-PB1 501 and H2-Kb-PB1703 in mice vaccinated with this viral vector. Interestingly, a similar pattern in reactivity to all the peptides under study was also measured in mice vaccinated in combination with MVA-M1 and MVA-NP viruses. In contrast to a non-replicating whole virus-based vaccine, these data show that CD8+ T cells to subdominant epitopes could be elicited when expressed by MVA vectors [25]. Considering the impact of multiple factors on establishing immunodominance hierarchies generated during the primary T cell responses, these results are also of interest because they show that a similar profile of CD8+ T cells against dominant and subdominant HLA-A2-restricted epitopes of internal influenza proteins was elicited by either MVA-based vaccines or influenza virus infection.

Although we could not assess the real contribution of HLA-A2-restricted CD8+ T cell responses for the presence of endogenous murine MHC class I molecules in AAD transgenic mice, it is conceivable that the A2-restricted subdominant epitopes on NP may only marginally contribute to survival against virus challenge. In addition to the contribution of CD8+ T cells specific to the H2-Db-NP366 epitope, anti-NP antibodies most likely conferred effective anti-viral activities by promoting the protection of mice against a lethal influenza virus challenge.

Figure 4. Protective effect against influenza virus challenge. Mice (7/group) were immunized twice with MVA vectors and challenged i.n. four weeks later with 2 LD50 of CA/09 virus. Body weight and survival were monitored for fourteen days after virus infection, and mice were sacrificed when body weight reached 75% of starting weight. One of three similar experiments is shown.

Figure 5. MHC class I-restricted recall responses following influenza virus challenge. Naive mice or mice vaccinated with two doses of the three-MVA virus combination four weeks earlier (6/group) were challenged i.n. under anesthesia with 2 LD50 of CA/09 virus. Seven days after challenge, mice were sacrificed and influenza-specific CD8+ T cell responses were measured in cells from bulk splenocytes by means of an ex vivo IFN-γ-ELISPOT assay with the indicated peptides. Bars represent the means ± SD of triplicate cultures. The data are representative of two independent experiments that gave similar results.
Fcr-mediated phagocytosis and complement-mediated lysis of virus-infected cells [39]. Here, we have been able to show that MVA-M1 vaccine alone can induce a protective immunity against influenza in AAD mice. This finding is consistent with previous studies showing that M1-based vaccines are capable of inducing protection against influenza in HLA-A2 transgenic mice that generate A2-M1<sub>10</sub>-specific CD8<sup>+</sup>T cells [40-42]. We could not detect M1-specific antibodies, as also reported previously in BALB/c mice immunized with the MVA-M1 vector [22]. Recent studies show that M1-specific antibodies elicited by DNA prime-vaccinia virus boost regimens based on M1 protein could, in the absence of specific T cells, confer protection (70% survival rate) after infection of BALB/c mice with 1.7 LD<sub>50</sub> of the A/PR/8 virus [19]. Thus, strategies to ameliorate the M1-specific antibody response induced by MVA-based vaccine alone or in combination with other antigens would be extremely beneficial for these promising viral vectors. Previous studies have shown that NP protein accumulates in lipid rafts at the apical cell membrane, even when expressed alone, whereas the M1 protein seems to bind cell membranes through electrostatic interactions [43,44]. Thus, different localization and interaction of the M1 protein with the cell membrane may affect antigen presentation to B cells, and explain the lack of induction of M1-specific antibodies, compared to the NP protein [45]. In this context, it would be interesting to determine if the fusion of M1 with portions of NP or other conserved antigens, such as the transmembrane and extracellular domains of M2 protein, would increase the partition of M1 into the cell membrane and thus improve the immunogenicity of MVA-M1 vectors expressing these chimeric constructs.

Finally, our data reveal that both M1- and NP-based MVA vaccines, regardless of whether they were applied individually or in combination, conferred protection against lethal virus challenge in AAD mice and clearly correlated with the high level of recall responses to the immunodominant epitopes in these animals. Although mouse models do not closely reflect human influenza virus infection, we can provide further evidence of the efficacy of NP- and M1-based influenza vaccines. We could not demonstrate a protective efficacy of an MVA vector expressing PB1, as also reported by others [19,22]. Nevertheless, the inclusion of PB1 in the vaccine may still help boost pre-existing immune T cells in the context of different haplotypes and thus contributes to virus clearance.

In summary, we show that MVA vectors expressing influenza proteins could induce high numbers of influenza-specific CD8<sup>+</sup>T cells to epitope peptides, which are conserved among different viral strains, and provide a significant level of protection against lethal challenge in AAD mice. Although we are still far away from the ‘one-shot for life,’ boosting of T cell responses to conserved viral antigens is, together with cross-reactive antibodies, an essential step toward a successful universal vaccine against influenza.

**Competing interests**

The authors declare that they have no competing interests.

**Acknowledgment**

We thank Andrea Giovannelli for assistance with the animal experiments.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was funded by the Ministry of Health, Italy [grant number RF-2010:2318269] and Fondazione Cariplo [grant number 2009-3594].

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