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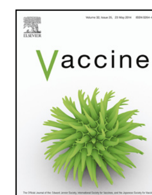

Activation of cross-reactive mucosal T and B cell responses in human nasopharynx-associated lymphoid tissue *in vitro* by Modified Vaccinia Ankara-vectored influenza vaccines

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ABSTRACT

Recent efforts have been focused on the development of vaccines that could induce broad immunity against influenza virus, either through T cell responses to conserved internal antigens or B cell response to cross-reactive haemagglutinin (HA). We studied the capacity of Modified Vaccinia Ankara (MVA)-vectored influenza vaccines to induce cross-reactive immunity to influenza virus in human nasopharynx-associated lymphoid tissue (NALT) *in vitro*. Adenotonsillar cells were isolated and stimulated with MVA vaccines expressing either conserved nucleoprotein (NP) and matrix protein 1 (M1) (MVA-NP-M1) or pandemic H1N1 HA (MVA-pdmH1HA). The MVA vaccine uptake and expression, and T and B cell responses were analyzed. MVA-vectored vaccines were highly efficient infecting NALT and vaccine antigens were highly expressed by B cells. MVA-NP-M1 elicited T cell response with greater numbers of IFNγ-producing CD4+ T cells and tissue-resident memory T cells than controls. MVA-pdmH1HA induced cross-reactive anti-HA antibodies to a number of influenza subtypes, in an age-dependent manner. The cross-reactive antibodies include anti-avian H5N1 and mainly target HA2 domain. *Conclusion*: MVA vaccines are efficient in infecting NALT and the vaccine antigen is highly expressed by B cells. MVA vaccines expressing conserved influenza antigens induce cross-reactive T and B cell responses in human NALT *in vitro*, suggesting the potential as mucosal vaccines for broader immunity against influenza.

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1. Introduction

Influenza continues to cause widespread morbidity and mortality, resulting in major challenges for healthcare systems [1,2]. 2009 pandemic H1N1 (pdmH1N1) influenza and the potential of pandemics by H5N1 or other avian influenza viruses highlight the need to find more effective preventative measures against these threats.

The most cost-effective public health intervention is vaccination. Current influenza vaccines principally induce humoral immunity against haemagglutinin (HA), which varies between virus strains, so the composition has to be reformulated each year. Recent studies suggest 2009 pdmH1N1 elicited cross-reactive memory B cell responses that produce antibodies against multiple influenza subtypes [3–7]. Current efforts focus on developing vaccines that induce broad immunity against influenza, either through T cell responses to conserved internal antigens or B cell response to cross-reactive HA. These include the novel influenza vaccine MVA-NP+ M1, using a replication-deficient viral vector (Modified Vaccinia Ankara, MVA) expressing the highly conserved internal proteins nucleoprotein (NP) and matrix protein 1 (M1) to boost memory T-cell responses [8]; and the use of a viral vector expressing pdmH1N1 HA to induce protective antibody response [9].

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As influenza virus causes disease by infecting nasopharynx mucosa, intranasal immunization provides an effective vaccination strategy. Live attenuated influenza vaccines (LAIV) have been used as intranasal immunization effectively in children, and has been shown to elicit lasting T and B-cell immunity (>1 year) [10]. The induction of local mucosal immunity by intranasal immunization critically relies on local immune organs–nasopharynx-associated lymphoid tissue (NALT). Adenoids and tonsils are major components of NALT and are known to be important induction sites for immunity against respiratory pathogens including influenza [11–14].

Recombinant replication-deficient viral vectors are capable of priming and boosting T cell responses against antigens they encode. Intranasal delivery of an adenovirus vectored vaccine expressing NP and M1 was shown in animal studies to induce stronger immune responses and greater protection than *via* intramuscular immunization [15]. MVA is one of the most studied viral vectors and has an excellent safety profile. MVA is highly attenuated and has been used to boost T-cell responses against HIV, tuberculosis and malaria in addition to influenza [16–18]. Recent studies in animal models showed MVA encoding influenza antigens could induce cross-reactive immunity [19,20]. Experimental use of MVA constructs *via* mucosal routes has been demonstrated to be efficient in generating protective immune responses to influenza and respiratory syncytial virus in mice [21,22].

Although animal models are frequently used to assess the immunogenicity of influenza vaccines, these are of limited utility since the animals used are naïve to influenza virus, and are not a good model for humans who have been exposed to influenza virus many times during lifetime, and have accumulated both B and T cell immunity to influenza as a result [23].

We previously studied the naturally acquired immunity including memory responses to influenza virus and *Streptococcus pneumoniae* protein antigens in human NALT tissue [7,24–26]. In this study, we have investigated the capacity of MVA-vectored influenza vaccines expressing either NP or HA to induce mucosal T and B cell immune response *in vitro* against influenza viruses in human NALT. We show that MVA vectored vaccines are very efficient in infecting the NALT *in vitro* and highly expressed in B cells and are able to induce cross-reactive T and B cell immunity to influenza viruses.

2. Methods

2.1. Patients and samples

Adenoids and tonsils were obtained from patients (range 2–35 years, $n = 55$) undergoing adenoidectomy and/or tonsillectomy due to upper airway obstruction. A venous blood sample was also obtained at time of surgery. Patients who had an immunodeficiency and who had received influenza vaccination were excluded from the study. The study was approved by Liverpool Paediatric Ethics Committee, and informed consent was obtained from each patient.

2.2. Recombinant MVA vaccines

The following recombinant MVA vectored vaccines were used, as well as non-recombinant MVA (nrMVA) as vector control. MVA-pH1HA expresses pandemic H1N1 HA under the control of the modified Vaccinia H5 promoter. MVA-NP-GFP expresses NP from A/Panama/2007/99 expressed from the Vaccinia p7.5 early/late promoter and GFP expressed from the fowlpox late FP4b promoter. Three PCR assays were used for quality control of MVA-pH1HA and MVA-NP-GFP respectively. For MVA-pH1HA, firstly, primers VVCF2 (CCATCGAGTGCGGCTACTAT) and J905 (GAGGGTTGTGTTAAA TTGAAAGCGAG) bind to the flanking regions of the MVA either side of the antigen insertion site and were used to confirm the presence of an insert of the expected size (1910 bp). Absence of the parental MVA sequence, which would have resulted in a PCR product of 637 bp with primers J459 (GCGACCTCATTTGCACTTTCTGGTTCG) and J1118 (GCTGAAGGGCGAGATCCACAAGGC) was also confirmed. Thirdly, primer J1267 (GTGAACAGCGTGATCGAGAA) that binds within the antigen sequence was used together with primer VVCF2 as before to confirm the presence of the expected antigen at the expected insertion site. Quality control testing also confirmed sterility of the virus prep while titration of plaque forming units gave the virus concentration in PFU/ml. For quality control of MVA-NP-GFP, the same procedures were followed and the 3 primer sets used were primers J506 (CTGATCACTAATTCCAAACCCACCCGC) and J459 (GCGACCTCATTTGCACTTT CTGGTTCG) (size 2105 bp), primers J459 and J481 (CTCCAAGCTGGACATCACCT CCCACAACGAG) (size 517), primers J230 (CGCCAAGCCCGAGGAAGTG) and J459 respectively. MVA-NP+M1 expresses NP and M1 from A/Panama/2007/99 as a fusion protein joined by a seven amino acid linker, from the Vaccinia p7.5 early/late promoter [8]. MVA-GFP expresses GFP from fowlpox late FP4b promoter. All insertions were made at the thymidine kinase locus of MVA.

2.3. Recombinant antigens

Purified recombinant HA0 (whole-length HA) of pdmH1N1 (A/California/04/2009), seasonal H1N1 (sH1N1) (A/H1N1/Brisbane/59/2007), seasonal H3N2 (sH3N2) (A/Brisbane/10/2007), avian H5N1 (aH5N1) (A/Vietnam/1203/2004) were obtained from BEI Resources (Manassas, VA). Recombinant HAs of pdmH1N1 and sH1N1 contain a C-terminal histidine tag and were produced in High Five™ insect cells using a baculovirus expression vector system. The HAs were purified by affinity chromatography and contain a trimerizing domain [27]. Recombinant HAs of sH3N2 and aH5N1viruses were glycosylated HA produced in Sf9 insect cells, and membrane-extracted from infected cells and purified under native conditions by affinity chromatography [28]. Recombinant HA1 and HA2 subunits (BEI Resources and SinoBiologicals) were his-tagged and produced using baculovirus expression system and purified by affinity chromatography. The proteins were re-folded into a soluble form and were shown to react to specific anti-HA1/HA2 antibodies by Western blotting. HA1 proteins were derived from the same subtype viruses as above for HA0. HA2 proteins were derived from pdmH1N1 (A/California/04/2009) and aH5N1 (A/Vietnam/1203/2004). The recombinant NP of A/PuertoRico/8/34/MountSinai (H1N1) containing a C-terminal histidine tag was produced similarly in a baculovirus system (SinoBiological).

2.4. Cell isolation and culture

Adenoidal and tonsillar tissues were checked for any signs of gross inflammation and/or necrosis prior to processing and any samples that exhibited either of these features were excluded. Mononuclear cells (MNC) were isolated from adenotonsillar tissues and cultured in RPMI cell culture medium following methods described previously [7,26,29].

2.5. Measurement of MVA infection

MVA expressing NP and GFP (MVA-NP-GFP) and MVA expressing GFP only (MVA-GFP) were used to determine the efficiency of MVA infection of different cells. Tonsillar MNC or PBMC were co-cultured with different doses of MVA-NP-GFP or MVA-GFP

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