MVA-NP+M1 vaccine activates mucosal M1-specific T cell immunity and tissue-resident memory T cells in human nasopharynx-associated lymphoid tissue

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Short title: vaccine induced mucosal T cell immunity

<u>Summary</u>: MVA-NP+M1 vaccine activates a substantial increase in anti-influenza M1-specific T cells including fast-reacting tissue-resident memory T cells in human nasopharynx mucosal tissue. MVA-NP+M1 is therefore a promising mucosal vaccine candidate with great potential for immediate local protection against influenza re-infection.

Word count: Abstract: 200. Text: 3498

Footnote:

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Funding: We also acknowledge funding support from SPARKS Medical Research, UK [15Liv01] and British Medical Association [HC Roscoe Award], and the Government Pharmaceutical Organization (GPO), Thailand [studentship]. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Conflict of interest: Sarah Gilbert is an inventor on patents covering MVA-NP+M1, filed and owned by the University of Oxford, and is a co-founder of and consultant to Vaccitech, a University of Oxford spin-out company which is undertaking advanced clinical development of viral vectored influenza vaccines.

Abstract

Increasing evidences support a critical role of $CD8^+$ T cell immunity against influenza. Activation of mucosal $CD8^+$T cells, particularly tissue-resident memory T(T_{RM}) cells recognizing conserved epitopes would mediate rapid and broad protection. Matrix protein 1(M1) is a well-conserved internal protein. We studied the capacity of Modified Vaccinia Ankara-vectored vaccine expressing nucleoprotein(NP) and M1(MVA-NP+M1) to activate M1-specific CD8+ T cell response including T_{RM} cells in nasopharynx-associated lymphoid tissue(NALT) from children and adults. Following MVA-NP+M1 stimulation, M1 was abundantly expressed in adenotonsillar epithelial cells and B cells. MVA-NP+M1 activated marked IFN-γ-secreting T cell response to M1 peptides. Using tetramer staining, we showed the vaccine activated a marked increase in $M1_{58-66}$-specific $CD8^+$ T cells in tonsillar mononuclear cells (MNC) of HLA-matched individuals. We also demonstrated MVA-NP+M1 activated a substantial increase in T_{RM} cells exhibiting effector memory T cell phenotype. Upon recall antigen recognition, M1-specific T cells rapidly undergo cytotoxic degranulation, release granzyme B and pro-inflammatory cytokines, leading to target cell killing. Conclusion: MVA-NP+M1 elicits a substantial M1-specific T cell response including T_{RM} cells in NALT, demonstrating its strong capacity to expand memory T cell pool exhibiting effector memory T cell phenotype, therefore offering great potential for rapid and broad protection against influenza reinfection.

Key words: Influenza, T cell immunity, vaccine, antigen-specific T cell, tissue-resident memory T cells (T_{RM}), nasopharynx-associated lymphoid tissue, cytotoxic T cell.

Introduction

Influenza still causes widespread morbidity and mortality, despite the available vaccines. Current influenza vaccines predominantly induce subtype-specific antibodies towards hemagglutinin (HA). As HA continuously mutates, vaccine composition needs to be updated every year, and vaccine efficacy varies considerably depending upon how well the vaccine strains match circulating viruses[1]. There is a need for more effective vaccines that confer broad immunity against influenza including those with potential to cause pandemics.

Although neutralizing HA-specific antibodies are considered the major protective responses[2], increasing evidence supports an important role for $CD8^+$ T cell-mediated immunity. In individuals experimentally infected with influenza, virus-specific cytotoxic T cell killing reduced virus shedding in absence of specific antibodies[3]. Pre-existing cytotoxic $CD8^+$ T cells were associated with decreased disease severity in patients infected with pandemic H1N1 virus[4].

The majority of influenza virus-specific $CD8^+$ T cells recognize epitopes shared among virus subtypes, including internal antigens nucleoprotein(NP) and matrix protein 1(M1)[5, 6], which are highly conserved with over 90% homology among different strains[7]. M1 protein plays a pivotal role in influenza virus replication[8, 9]. Activation of these T cell responses would mediate a broadly cross-reactive protection[10]. A number of novel T cell-based influenza vaccines are being developed[11], including Modified Vaccinia Ankara virus (MVA)-vectored vaccines[12-14]. MVA-NP+M1 is one of the promising vaccine candidates, showing activation of antigen-specific T cell responses in peripheral blood following parenteral immunization[15, 16].

Tissue-resident memory T cells(T_{RM}) reside in tissues and provides rapid response against re-infections at body surfaces[17]. T_{RM} are anatomically positioned to quickly respond to local infection. Animal models showed T_{RM} made critical contributions to protective immunity against local challenges which was much more effective than recirculating memory T cells [18-20]. A vaccine strategy that enables establishment and/or expands mucosal T_{RM} would have enormous potential for immediate protection against reinfection, offering more effective disease control [21].

Since influenza virus infects through nasopharyngeal mucosa, local intranasal vaccine delivery that activates cross-reactive mucosal T cell immunity including T_{RM} offers an attractive strategy. Intranasal live attenuated influenza vaccine(LAIV) were shown to induce local and systemic antibodies and T cell immunity in children[22-24]. Aerosol delivery of a candidate universal influenza vaccine induced local cellular responses associated with partial protection against heterosubtypic influenza A in pigs[25]. Intranasal immunization relies on local nasopharynx-associated lymphoid tissue(NALT) to induce T and B cell responses. Adenotonsillar tissues are major components of human NALT known to be important induction sites for immunity against respiratory pathogens[26-28].

We previously demonstrated cross-reactive memory B cell responses were primed following 2009 pdmH1N1 infection[29] and activation of NP-specific T cell response by MVA-NP+M1 in human NALT[30]. As M1 contains major immuno-dominant CD8+ T cell epitopes and HLA-A2 is among the most common HLA alleles(20-30%)[31], we examined HLA-A2 restricted $M1_{58-66}$-specific $CD8^+$ T cell responses in adenotonsillar tissue following MVA-NP+M1 stimulation. We show MVA-NP+M1 elicits marked increases in M1-specific CD8+ T cells including T_{RM} that exhibit rapid degranulation and target cell killing upon recall antigen recognition.

Methods

Patients and samples

Tonsillar tissues and peripheral blood samples were obtained from immune-competent children and adults (age 2-34 years) undergoing tonsillectomy due to upper airway obstruction. Tissue samples were obtained from Alder Hey Children’s Hospital and Aintree

University Hospital in Liverpool, UK. Demographic information of studied patients was summarized in Table 1. Patients who had any known immunodeficiency were excluded. Grossly inflamed tonsillar tissues were also excluded. Ethical approval was obtained (REC No: 14/SS/1058) and informed consent was obtained in all cases.

Vaccines and peptides

MVA-NP+M1 is Modified Vaccinia Ankara (MVA) virus expressing NP and M1 from A/Panama/2007/99 as a fusion protein joined by a seven amino acid linker, from Vaccinia p7.5 early/late promoter. MVA-wt was non-recombinant MVA used as a vector control. 9-mer conserved peptides of influenza M1 (BEI resources)(Table 2) were reconstituted in 50% acetonitrile or Dimethyl Sulfoxide(DMSO) following manufacturer's instruction. 10 or 11 peptides were pooled at a concentration of 0.1 mg/ml per peptide. $M1_{58-66}$(GILGFVFTL)(IBA GmbH) was reconstituted in DMSO(50%) at final concentration 1 mg/ml.

Fluorescence-labeled antibodies and M1-tetramer

The following fluorescence-labeled antibodies were used in flow-cytometry including those to HLA-A2, CD19, CD3, CD11c, CD123, CD8, CD69, granzymeB, CD107a, IFNγ, TNFα, IL-2, CD20, CD38, CD27, IgD, CCR7, CD45RA- and CD103 (BD Bioscience or Biolegend). Anti-M1 antibody(abcam) was conjugated with PE using LYNX conjugation(Bio-rad) for measuring M1 protein expression. HLA-A02*01-GILGFVFTL($M1_{58-66}$)-PE tetramer(MBL), termed as "M1-Tm" was used for staining M1-specific $CD8^+$ T cells.

Cell isolation

Tonsillar mononuclear cells (MNC) were isolated using density gradient centrifugation as described previously[32, 33]. Tonsillar MNC were resuspended in RPMI-1640 containing HEPES, L-glutamine, 10% heat-inactivated fetal bovine serum(FBS), 100 U/ml penicillin and 100 μg/ml streptomycin(Gibco), termed as "complete RPMI medium". MNC were screened for HLA-A2 type by flow-cytometry.

Measurement of M1 expression in tonsillar MNC

Tonsillar MNC were stimulated with either MVA-NP+M1 at 1.0 multiplicity of infection (MOI) and incubated for 18-20 hours. MNC were stained for epithelial cell markers including pan-cytokeratin and epithelial cellular adhesion molecule(EpCAM), and CD19/CD4/CD11c/CD123, followed by intracellular staining for M1 expression using anti-M1 antibody. B cell subsets were determined by fluorescence staining and identified as memory($CD19^+CD20^+CD38^-CD27^+IgD^-$), naïve($CD19^+CD20^+CD38^-IgD^+CD27^-$) and germinal center(GC) B cells ($CD19^+CD20^+CD38^+$)[34].

Cell stimulation for T cell assays

Tonsillar MNC were co-cultured with either MVA-NP+M1 or MVA-wt at $1x10^5$ pfu/ml. Cell culture in complete RPMI medium was supplemented with 2% autologous human plasma (aHP). Tonsillar MNC were incubated for 7 days before any further experiments. Non-HLA typed tonsillar MNC were used for pooled-peptides stimulation and IFN-γ ELISPOT assay, whereas MNC from HLA-A2+ individuals were used for M1-specific $CD8^+$ T cell response by tetramer staining.

IFN-γ ELISPOT

At day-7 following culture, MVA-NP+M1-stimulated cells were rested in RPMI for 2 days followed by IFN-γ ELISPOT assay(eBioscience). ELISPOT plate(Millipore) was coated with anti-IFN-γ antibody overnight. $2x10^5$ cells stimulated with M1 peptide pools(10 µg/ml per peptide) were seeded in plate wells. Cells without stimulation were as negative control, and cells stimulated with SEB (BEIResources) as positive control. The plate was incubated for 24 hours, followed by addition of anti-IFN-γ detection antibody and Avidin-horseradish peroxidase. Spots were developed by adding 3-amino-9-ethyl carbazole(Sigma) and counted by EliSpot Reader.

Detection of M1$_{58\text{-}66}$-specific CD8^{+} T cells and T$_{RM}$ cells

For flow-cytometric analysis of M1-Tm^{+} CD8+ T cells and their phenotypes in tonsillar tissue, freshly isolated tonsillar MNC, or MNC following co-incubation with M1$_{58\text{-}66}$ peptide for 2 days (to expand M1-Tm^{+} cells) were stained with HLA-A02*01-M1$_{58\text{-}66}$-PE tetramer. HLA-A02*01 control tetramers including HLA-A02*01-HPV16 E7(-YMLDLQPET) and HLA-A02*01-negative control tetramer (-ALAAAAAAV)(MBL) were used. The specific detection of M1-Tm^{+} cells in tonsillar MNC was confirmed by positive staining in CD8+ T cells only by M1-Tm tetramer, and negative staining by control tetramers in MNC following M1-peptide stimulation(data not shown). Tonsillar MNC were also co-cultured with MVA-NP+M1, followed by analysis of M1-Tm^{+} cells. For detection of M1-specific T$_{RM}$, in addition to the above, MNC were co-stained with anti-CD103, -CD69, -CD45RA and –CCR7 antibodies.

Measurement of T cell proliferation

Tonsillar MNC were labeled with Carboxyfluorescein succinimidyl ester(CFSE, 5μM) (Invitrogen)[35]. CFSE-labeled cells were resuspended in RPMI supplemented with 2%aHP before stimulation with 1x10^{5} pfu/ml of MVA-NP+M1 for 5 days. Cells were then stained for CD8 and M1-Tm, followed by flow-cytometry.

Detection of CD107a expression and intracellular cytokines

Following 7-day MVA-NP+M1 stimulation, tonsillar MNC were pulsed with 0.25 μg/ml M1$_{58\text{-}66}$ peptide and co-cultured with anti-CD107a antibody in the presence of brefeldin A and monensin(eBioscience). Cells were collected and stained for CD8 and M1-Tm, and intracellular cytokines followed by flow-cytometry.

Cytotoxic killing assay

Isolated CD8^{+} T cells following MVA-NP+M1 stimulation were co-cultured with M1$_{58\text{-}66}$-pulsed B cells as described previously[36]. Briefly, autologous B cells were isolated from cryopreserved tonsillar MNC and incubated overnight with 40ng/ml recombinant IFN-γ

(Peprotech). B cells were then labeled with either 0.02 μM(T_{low}) or 0.2 μM (T_{high}) of CFSE for 15 min. T_{low} were pulsed with 5 μg/ml $M1_{58\text{-}66}$ for 45 min. Both T_{low} and T_{high} were adjusted to $2x10^5$ cells/ml and mixed at ratio 1:1. For effector cells, isolated $CD8^+$ T cells following stimulation were adjusted to $4\text{-}10x10^6$ cells/ml before 2-fold serial dilutions were made (1:1 to 1:32). $CD8^+$ T cells were then co-cultured at different ratios with mixed T_{low} and T_{high} cells for 6 hours. Mixed T_{low} and T_{high} cells only (without $CD8^+$ T cells) were cultured as negative control. Cells were harvested and stained with LIVE/DEAD Far red (Invitrogen) for 30 min before staining for CD8 and M1-Tm.

Flow cytometry

Fluorescence-labeled cells were analyzed using BD FACScalibur with CellQuest or Celesta with FACS DiVa (BD) and analyzed using FlowJo 8.7 software.

Statistical analysis

For two-group comparisons, based on normality of data, parametric paired-t test, nonparametric Wilcoxon matched-pairs signed rank test and nonparametric Mann-Whitney test were performed using GraphPad Prism. $p<0.05$ was considered as statistically significant.

Results

M1 antigen was highly expressed in NALT following MVA-NP+M1 stimulation

To determine whether M1 antigen was expressed in tonsillar cells following MVA-NP+M1 stimulation, we examined M1 expression in tonsillar MNC by intracellular M1 staining. As shown in Figure 1a and 1b, following stimulation, M1 was abundantly expressed in tonsillar epithelial cells (Mean±SEM: 34.5±3.2%) and B cells(35.2±7.55%), but only a small number of T cells(2.3±0.6%). Among B cells, M1 expression was detected in memory(55.8±2.2%), naïve(48.7±2.5%), and germinal center(GC) B cells(22.7±0.9%) respectively(data not shown). Among tonsillar dendritic cells(DC), M1 expression was shown in myeloid DC(21.2±3.2%) and plasmacytoid DC(22.0±7.1%)(Figure 1b). As a control, no M1 expression was detected in any cell types following MVA vector only stimulation.

MVA-NP+M1 elicited mucosal M1-specific T cell responses.

Having shown abundant M1 expression in tonsillar MNC, we investigated whether MVA-NP+M1 activated M1-specific T cell responses. Following MVA-NP+M1 stimulation, tonsillar MNC were co-incubated with 9-mer M1-peptide pools(Table 2) followed by IFN-γ ELISPOT. A marked increase in IFN-γ-secreting cells was found in MNC stimulated by MVA-NP+M1, as compared to that by MVA vector alone(Figure 1c+d, $p<0.05$). Subsequent flow-cytometry revealed the increase in IFN-γ-secreting cells following M1-peptides re-stimulation was predominantly from $CD8^+$ T cells but not from $CD4^+$ T cells(Figure 1e), with a mean increase of 0.27±0.05% of IFN-γ-secreting cells (% of CD8+ T cells). This suggests MVA-NP+M1 stimulation activates a marked M1-specific T cell response.

To confirm this, we examined M1-specific $CD8^+$ T cell response using HLA-A2-restricted $M1_{58\text{-}66}$-specific tetramer(Tm) staining in HLA-matched individuals(Figure 2a). Frequencies of $M1\text{-}Tm^+$ cells in freshly isolated MNC were generally low(median 0.10%). MVA-NP+M1 stimulation elicited a marked increase in $M1\text{-}Tm^+$ cells(median 0.37%), compared to that by MVA vector or medium control (Figure 2b, $p<0.001$). When MVA-NP+M1 activated $M1\text{-}Tm^+$ cell response was compared among different age groups(Table 1), an age-dependent increase was shown in $M1\text{-}Tm^+$ cell response. Children<4 years in general showed a low/modest response, whereas older children and adults demonstrated stronger responses(Figure 2c).

Further analysis with CFSE cell tracing demonstrated MVA-NP+M1 activated a proliferative $M1\text{-}Tm^+$ cell response in tonsillar MNC, compared to that by MVA vector only(Figure 2d, $p<0.05$).

MVA-NP+M1 elicited M1-specific T_{RM} response

To determine whether there were M1-specific T_{RM} in NALT and if MVA-NP+M1 activated an increase in T_{RM}, we studied tonsillar MNC from HLA-matched subjects(age 5-24 years) by co-staining T_{RM} markers and M1-tetramer.

As frequencies of M1-Tm+ cells in *ex vivo* tonsillar tissue were low, we used M1-specific peptide to enrich M1-Tm+ cells in tonsillar MNC (and in PBMC) by co-incubation with $M1_{58-66}$ peptide for 2 days. The phenotypes of expanded M1-Tm+ cells following peptide stimulation showed no difference to freshly isolated MNC(data not shown). In tonsillar MNC, there were 25.1±3.2%(mean±SEM) of M1-Tm^+ cells expressing $CD103^+$ therefore identified as M1-specific T_{RM}, and most of them were $CD103^+CD69^+$ T_{RM} (Figure 3a+e). There were also 38.1±3.6% of M1-Tm^+ cells expressing CD69 but not CD103 ($CD103^-CD69^+$). Of M1-Tm^+ cells in MNC, around 64% were of effector memory T cell phenotype($CD45RA^-CCR7^-$) (Figure 3b+f). Among M1-Tm^+ cell subsets, the majority (64.2±8.4%) of $CD103^+CD69^+$ T_{RM} cells were of effector memory T cell phenotype, compared to 42.6±6.1% and 14.4±2.5% respectively for $CD103^-CD69^+$ and $CD103^-CD69^-$ subsets (Figure 3i). By contrast, in PBMC from the same subjects, none of M1-Tm^+ cells expressed CD103 (thus non-T_{RM} cells), and only ~20% were of $CD45RA^-CCR7^-$ effector memory phenotype, with the majority were of $CD45RA^+CCR7^-$ phenotype (Figure 3b+f).

Following MVA-NP+M1 stimulation, there was a substantial increase in M1-Tm^+ cells (6-18 fold-increase) including both $CD103^+$ and $CD103^-$ cell subsets, and a large majority (~90%) expressed $CD45RA^-CCR7^-$ phenotype(Figure 3c+g+h). Of interest, in CD103+ T_{RM} cells, there was a marked increase in $CD103^+CD69^-$ subset which accounted for ~75% of CD103+T_{RM}, whereas ~25% were $CD103^+CD69^+$ (Figure 3c+g). This contrasted with freshly isolated MNC or M1-peptide expanded MNC in which $CD103^+$ cells were primarily $CD103^+CD69^+$. Further, when the memory phenotypes were analyzed, more $CD103^+CD69^-$ T_{RM} cells(mean: 86.1%) exhibited an effector memory phenotype ($CD45RA^-CCR7^-$), than $CD103^+CD69^+$ (65.6%) or $CD103^-CD69^+$ (42.2%) T_{RM} subsets(Figure 3j). When PBMC from the same subjects were analyzed, a marked increase in M1-Tm^+ cells was also seen, but these cells in PBMC were largely $CD103^-CD69^-$ non-T_{RM} cells (Figure 3c+g).

MVA-NP+M1 activated M1-specific CD8+ T cells exhibited cytotoxic functions and killing property.

To determine whether MVA-NP+M1-activated M1-specific $CD8^+$ T cells in tonsillar MNC were functionally active, we examined the expression of cytotoxic molecules and cytokines of M1-Tm^+ cells. At day-7 following vaccine stimulation, the M1-Tm^+ cells expressed a high level of granzyme-B(Figure 4a+b). Tonsillar MNC were subsequently pulsed with $M1_{58-66}$ peptide followed by detection of surface CD107a(marker for degranulation) and cytokine expression. Both CD107a and IFN-γ expressions were markedly upregulated in M1-Tm^+ cells after $M1_{58-66}$ peptide pulsing(Figure 4c). Kinetics of CD107a and IFN-γ expression were further studied and a similar pattern was shown for both(Figure 4d+e). Notably, a more rapid upregulation in expression of CD107a than IFN-γ was seen. At one hour following peptide pulsing, ~40% of M1-Tm^+ cells expressed CD107a, compared to 10% producing IFN-γ($p<0.05$). Both surface CD107a expression and IFN-γ production appeared to peak after 3 hours(Figure 4d+e). IFN-γ and TNF-α were abundantly expressed in M1-Tm^+ cells following peptide pulsing(Figure 4f+g). Figure 4h summarized frequencies of M1-Tm^+ cells expressing different cytokine profiles, with the most frequently detected M1-Tm^+ cells co-expressing CD107a with IFN-γ and TNF-α(45%). Some M1-Tm^+ cells(3%) were shown to co-express CD107a and three cytokines IFN-γ, TNF-α and IL-2(Figure 4h).

We further investigated whether M1-Tm^+ cells were capable of cytotoxic killing of target cells. Following MVA-NP+M1 stimulation, isolated $CD8^+$ T cells (as effector T cells:E) were co-cultured with $M1_{58-66}$ peptide-pulsed target B cells(T), followed by measurement of target cell lysis using flow-cytometry. As demonstrated in Figure 5a, there was a marked decrease in peptide-pulsed target B cells(T_{low}), while no decrease in B cells without peptide-pulsing(T_{high}) following co-culture with effector T cells, indicating M1-specific target cell lysis. In all the three samples tested, the increase in target cell lysis correlated well with the increase in effector to target cell (E/T) ratio(Figure 5b).

Discussion

Since intranasal vaccination is considered an effective vaccination strategy against respiratory pathogens[22-24], we investigated the potential of MVA-NP+M1 as a mucosal vaccine to activate anti-influenza T cell responses in human NALT. We demonstrated MVA-NP+M1 activates a prominent M1-specific cytotoxic T cell response with a marked increase in M1-specific T_{RM} cells.

Following MVA-NP+M1 stimulation, we showed M1 antigen was highly expressed in both tonsillar epithelial cells and B cells. This suggest MVA-NP+M1 has the capacity to efficiently infect tonsillar cryptal epithelium and present M1 antigen. Tonsillar tissue has a reticular crypt epithelium containing both epithelial and non-epithelial immune cells. An efficient infection of epithelium by MVA-vectored vaccine would provide a favorable environment for the vaccine uptake and antigen presentation. Memory B cells, representing a major non-epithelial immune cell subset, were mainly found within intraepithelial areas and have a strong capacity to present antigen directly to T cells, owing to the constitutive expression of co-stimulatory molecules[37-39]. The unique anatomical localization of memory B cells in intraepithelial areas, together with the strong antigen-presenting capacity has been considered critical for the prompt and robust memory antibody responses[37]. It is therefore possible that memory B cells are infected by the MVA vaccine virus and efficiently present the vaccine antigen (e.g. M1) to memory T cells, contributing to activation of memory T cells in tonsillar MNC. Dendritic cells may also contribute to vaccine uptake and antigen processing, as a significant proportion of myeloid DC and plasmacytoid DC showed M1 expression consistent with previous report[40].

With IFN-γ ELISPOT assay, we demonstrated MVA-NP+M1 activated a marked increase in IFN-γ-secreting $CD8^+$ T cells specific to conserved M1 epitopes. Further, using $M1_{58-66}$-specific tetramer staining, we showed MVA-NP+M1 stimulation elicited a marked increase in $M1\text{-}Tm^+$ T cells in tonsillar MNC from HLA-matched individuals, particularly in older children and adults. $M1_{58-66}$-specific CD8+ T cells has been shown previously to protect against influenza infection in HLA-A2 transgenic mice[41]. Our results therefore provide evidence in support of the capacity of MVA-NP+M1 to elicit M1-specific $CD8^+$ T cell responses with the potential for protection against influenza in human nasopharynx.

Recent research supports a critical role of T_{RM} cells in providing a rapid protection against influenza. T_{RM} in human lungs were shown to mount a rapid response and kill influenza-infected epithelial cells and contribute to protection[42, 43]. Using M1-tetramer and CD103/CD69 co-staining, we demonstrated the presence of $CD103^+$ M1-specific CD8+ T_{RM} cells in tonsillar tissue which were expanded by M1-specific peptide. Among M1-Tm^+ cells, there were both $CD103^+CD69^+$ and $CD103^-CD69^+$ T_{RM} subsets. Similar to a previous study on EBV-specific T_{RM} in tonsillar tissue [44], M1-specific $CD103^+$ cells were largely restricted to $CD69^+$ cells, and a large proportion of these T_{RM} cells were of effector memory T cell phenotype. It was shown previously that $CD103^+CD69^+$ T_{RM} preferentially localized to tonsillar epithelial surface, whereas $CD103^-CD69^+$ cells largely localized in extrafolicular regions[44]. Our results therefore support the presence of M1-specific T_{RM} cells in tonsillar epithelium, derived from memory T cells primed by previous influenza infection. These cells largely exhibit effector memory T cell phenotype with the ability to mount a fast response to re-infection.

Following MNA-NP+M1 stimulation, there was an increase in M1-specific T_{RM} ($CD103^+$) as well as non-T_{RM} cells ($CD103^-$) in tonsillar MNC. Interestingly, of $CD103^+$ T_{RM} cells, the majority were $CD103^+CD69^-$ whereas only ~25% were $CD103^+CD69^+$ cells which were predominant in unstimulated tonsillar MNC. It would be interesting to know whether there is any functional difference between $CD103^+CD69^-$ and $CD103^+CD69^+$ subsets in future studies. The fact that a large majority of $CD103^+CD69^-$ cells exhibited effector memory T cell phenotype indicates they have the capacity to respond to re-infection rapidly. These results suggest that MVA-NP+M1, if used as an intranasal vaccine, would be able to elicit a proliferative response of T_{RM} cells, to expand T_{RM} memory T cell pool in NALT, and offer rapid protection against influenza infection in the nasopharynx. MVA-NP+M1 most likely acts

by boosting pre-existing memory CD8+ T cells, but not by inducing de novo M1-specific T cells, as tonsillar MNC depleted of memory T cells(CD45RO+) failed to show any M1-Tm+ cells following MVA-NP+M1 stimulation(data not shown).
As a comparison, we also analyzed M1-Tm^+ T cells in PBMC, and demonstrated the absence of T_{RM} ($CD103^+CD69^+$) cells in PBMC before and after the vaccine stimulation. This supports the concept that CD103+ T_{RM} cells are retained in peripheral tissue but not present in the circulation. Local mucosal vaccination may therefore offer distinctive advantage in expanding antigen-specific T_{RM} cells in local tissues for rapid protection.

It is generally thought that cytotoxic $CD8^+$ T cells exert their effector activities to limit virus infection and disease severity[6, 10] through degranulation, cytotoxic molecule release and pro-inflammatory cytokines[45]. Here we demonstrated that M1-Tm^+ cells activated by MVA-NP+M1 expressed a high level of granzyme B, which were subsequently released upon recognition of $M1_{58-66}$ peptide, along with rapid upregulation of surface CD107a expression. In addition, many M1-Tm^+ cells co-expressed CD107a with IFN-γ and TNF-α, suggesting they produce both cytotoxic effector molecules and inflammatory cytokines upon antigen-specific recognition. IFN-γ and TNF-α are potent pro-inflammatory cytokines and important in anti-viral activity. In addition to these two cytokines, some of these cells also co-expressed IL-2, which may exhibit more potent cytotoxic functions [46, 47]. Although $CD4^+$ rather than $CD8^+$ T cells are the main source of IL-2, a small number of $CD8^+$ T cells can secrete IL-2 after receiving costimulatory signals, providing proliferation and survival signals to themselves or other cytotoxic T cells[45].

The kinetics of CD107a expression correlated well with that of cytokine (IFN-γ) production in the M1-Tm^+ cells. The rapid upregulation of surface CD107a expression (i.e. degranulation) in M1-Tm^+ cells upon specific antigen recognition suggests these M1-specific $CD8^+$ T cells, including T_{RM}, may mount an immediate cytotoxic response against influenza. Finally, using M1-specific peptide pulsed tonsillar B lymphocytes as target cells for the effector T cell function, we showed MVA-NP+M1-activated M1-Tm^+ cells possessing marked cytotoxic killing activity capable of target cell lysis.

In conclusion, we demonstrate MVA-NP+M1 activated a M1-specific mucosal $CD8^+$ T cell response including a substantial increase in T_{RM} cells. These M1-specific T cells were predominantly of effector memory T cell phenotype, exhibiting a high level of cytotoxic markers and producing pro-inflammatory cytokines leading to specific killing of target cells upon antigen recognition. Our results suggest this novel vaccine expands M1-specific T_{RM} cell pool and activates cytotoxic T cell responses to the conserved antigen, therefore offering great potential as an effective mucosal vaccine for fast and broad protection against re-infection of influenza virus in humans.

Acknowledgement. We thank the patients who took part in the study and the theatre staff in the Liverpool Children's Hospital and Aintree University Hospital for helping the collection of samples. We also acknowledge funding support from SPARKS Medical Research, UK [15Liv01] and British Medical Association [HC Roscoe Award], and the Government Pharmaceutical Organization (GPO), Thailand [studentship]. This work was also supported by the National Institute for Health Research (NIHR) Alder Hey Clinical Research Facility. The views expressed are those of the authors and not necessarily those of the funders, NHS or the UK Department of Health. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The following reagent was obtained through BEI Resources, NIAID, NIH: Peptide Arrays, Peptides for Expected Conserved MHC Class I Epitopes of Influenza Virus A Proteins, NR-2667.

Author Contributions: Conceived and designed the experiments: SP, MA, PM, SG, QZ. Performed the experiments and analysis: SP, MA, QZ. Contributed clinical samples, vaccines/materials: RS, MK, SL, TL, SG. All contributed to the writing of the manuscript.

Table 1 Study subjects information

Sample		Average Age (Range)	n
Children	Group 1	2.5 (2-3.5)	6
	Group 2	5.7 (4-9)	12
Adults		20.6 (16-34)	9

Table 2 List of 9-mer peptides of conserved MHC class I binding epitopes from M1 of influenza A viruses (NR-2667, BEI resources)

Influenza proteins	Pool No.	Peptide No.	Amino acid sequences (9)	HLA restriction
M1	1	1	29-EDVFAGKNT-37	HLA-A*03
		2	31-VFAGKNTDL-39	HLA-A*2402, HLA-B*08
		3	37-TDLEALMEW-45	HLA-A*01
		4	49-RPILSPLTK-57	HLA-A*03
		5	51-ILSPLTKGI-59	HLA-A*0201
		6	56-TKGILGFVF-64	HLA-A*02
		7	58-GILGFVFTL-66	HLA-A*02, HLA-A*2402
		8	60-LGFVFTLTV-68	HLA-A*02
		9	66-LTVPSERGL-74	HLA-A*02
		10	68-VPSERGLQR-76	HLA-A*02
	2	11	71-ERGLQRRRF-79	HLA-A*02
		12	75-QRRRFVQNA-83	HLA-A*02
		13	76-RRRFVQNAL-84	HLA-A*02
		14	122-GALASCMGL-130	HLA-B*35
		15	123-ALASCMGLI-131	HLA-B*35
		16	124-LASCMGLIY-132	HLA-B*35
		17	126-SCMGLIYNR-134	HLA-B*35
		18	177-NRMVLASTT-185	HLA-A*0301, HLA-A*11
		19	179-MVLASTTAK-187	HLA-A*0301, HLA-A*11
		20	180-VLASTTAKA-188	HLA-A*0301, HLA-A*11
		21	181-LASTTAKAM-189	HLA-A*0301, HLA-A*11

Figure legends

Figure 1. Expression of M1 in tonsillar MNC following MVA-NP+M1 stimulation, and T cell responses to conserved M1 peptides. M1 protein expression was examined in tonsillar MNC following either MVA-NP+M1 or MVA-wt stimulation for 18 hours. a) Representative flow cytometric histograms showed the expression of M1 protein in tonsillar epithelial cells and B cells following stimulation by MVA-NP+M1 (red line) as compared to MVA-wt (black line). b) Bar charts demonstrated the percentages of M1 expression in epithelial cells, B cells, plasmacytoid dendritic cells (pDC), myeloid dendritic cells (mDC) and T cells following MVA-NP+M1 stimulation as compared to MVA-wt (n=3, Means and SEMs are shown). Following MVA-NP+M1 stimulation and cell resting, the frequency of IFN-γ-secreting T cells upon restimulation by conserved M1 peptide pools were enumerated by IFN-γ-ELISPOT assay. c) Representative figures showed spots (as implied to IFN-γ-secreting cells) in MVA-NP+M1-stimulated as compared to MVA-wt-stimulated MNC before and after restimulation by M1 peptide pools. d) Comparison of frequency of IFN-γ-spot-forming cells (SFC/million) between MVA-NP+M1 and MVA-wt-stimulated MNC against M1 peptide pools (n=7, * $p<0.05$, Wilcoxon signed rank test). SFC frequency as indicated was obtained by subtracting background SFC from cells without peptide restimulation. e) Representative dot plots showed a higher frequency of IFN-γ-producing $CD8^+$ T cells than $CD4^+$ T cells following restimulation by M1 peptide pools in MVA-NP+M1-stimulated MNC (one of 3 representative samples was shown).

Figure 2. $M1_{58-66}$-specific $CD8^+$ T cells activated by MVA-NP+M1. $M1_{58-66}$–specific $CD8^+$ T cells (M1-Tm^+) were determined using M1 tetramer staining in HLA-A2+ subjects after 7-day culture of tonsillar MNC with MVA-NP+M1, MVA-wt or medium control. a) Gating strategy for analysis of M1-Tm^+ cells. b) MVA-NP+M1 activated an increase of M1-Tm^+ cells in children (black open circle) and adults (red open circle) compared to MVA-wt (Wilcoxon signed rank test, n=27, ***$p<0.001$). c) Comparison of the frequency of M1-Tm^+ cells among different age groups (* $p<0.05$, **$p<0.01$) (medians with interquartile ranges are shown). d) Gating on M1-Tm^+ cells, representative histogram showed M1-Tm^+ cell proliferation was activated by MVA-NP+M1 (blue line) as compared to MVA-wt control (grey shaded). e) Proliferation of M1-Tm^+ cells (%$CFSE_{low}$) following stimulation of tonsillar MNC by MVA-NP+M1 as compared to MVA-wt control (n=3, *$p<0.05$, Wilcoxon signed rank test).

Figure 3. **MVA-NP+M1 activated M1-specific T_{RM} response in tonsillar MNC.** Representative dotplots (gated on M1–Tm+ $CD8^+$ T cells only) demonstrating the presence of pre-existing $M1_{58-66}$–specific T_{RM} ($CD103^+CD69^+$) in M1-peptide expanded tonsillar MNC and PBMC (a) and substantially increased numbers of both $CD103^+$ and $CD103^-$ M1–Tm+ cells following MVA-NP+M1 stimulation at day 7, particularly the increase in $CD103^+CD69^-$ subset in tonsillar MNC(c). This contrasted with the findings in PBMC showing the absence of $CD103^+CD69^+$ T_{RM} cells in both M1-peptide expanded(a+e) and MVA-NP+M1-stimulated PBMC (c+g). Memory phenotypes of M1–Tm+ cells were examined using CCR7 & CD45RA markers in tonsillar MNC compared to PBMC (b+f:M1-peptide-expanded and d+h:MVA-NP+M1-stimulated). T_{RM} and Non-T_{RM} subsets (e & g) and their memory phenotypes (f & h)

of M1–Tm+ cells in tonsillar MNC and PBMC following M1-peptide and MVA-NP+M1 stimulation were summarized (e-h). M1-Tm+ cell memory phenotypes in different T_{RM} and Non-T_{RM} subsets in tonsillar MNC following M1-peptide (i) or MVA-NP+M1 stimulation (j) were compared (*p<0.05, **p<0.01 compared to $CD103^{-}CD69^{-}$ non-T_{RM} cells, n=5).

Figure 2. Cytotoxic molecule and pro-inflammatory cytokine expression profiles of M1 specific $CD8^+$ T cells. Tonsillar MNC were stimulated by MVA-NP+M1 for 7 days followed by detection of M1-Tm^+ cells and expression of cytotoxic molecules. Tonsillar MNC were subsequently pulsed with $M1_{58\text{-}66}$ peptide for 6 hours followed by detection of surface CD107a and intracellular cytokines. (a & b) MVA-NP+M1 activated M1-Tm^+ cells expressing high level of granzyme B as compared to MVA-wt alone (a: representative plots; b: n=8, *p<0.05). c). Following M1 peptide pulsing, both surface CD107a and intracellular IFN-γ were highly expressed in M1-Tm^+ as compared to the low level in M1-Tm^- cells (n=8 and 13 respectively, ****p<0.0001). d) Representative dot plots and e) the kinetics curves showed the co-expression of surface CD107a and intracellular IFN-γ in M1-Tm^+ cells following peptide pulsing. At 1 hour, the percentages of $CD107a^+$ cells were significantly higher than those of IFN-γ^+ cells (n=4, *p<0.05, paired-t test). Means and SEMs were shown at each time point. (f & g) Representative dot plots showed the high level of expression of IFN-γ (f) and TNF-α (g) in MVA-NP+M1 activated M1-Tm^+ cells. (h) Pie and bar charts demonstrated a functional profile of M1-Tm^+ cells in MVA-NP+M1 activated tonsillar MNC following by 6-hour re-stimulation with a $M1_{58\text{-}66}$ peptide, showing the co-expression of CD107a and 3 cytokines, IFN-γ, TNF-α and IL-2 (one of 2 representative samples was shown).

Figure 5. Specific killing capacity of $M1_{58\text{-}66}$-specific $CD8^+$ T cells. Isolated $CD8^+$ T cells following MVA-NP+M1 stimulation were co-cultured at different ratios with autologous B cells labeled with low (T_{low}) and high CFSE intensities (T_{high}). T_{low} were either pulsed with $M1_{58\text{-}66}$ or without pulsing, while T_{high} were without pulsing. a) Representative dotplots and histogram demonstrating the decrease in target cells (T_{low}) following M1-peptide pulsing (green gate or middle peak), as compared to non-pulsing controls (grey shaded), indicating M1-specific target cell killing. b) Correlations between % of M1-specific target cell lysis and effector to target cells (E/T) ratio in three subjects were shown. E refers to effector number of M1-Tm^+ cells of isolated $CD8^+$ T cells, whereas T refers to number of target T_{low} cells. The proportion of M1-Tm^+ cells in the total isolated CD8+ T cells ranged from 1 to 4%.

Supplemental figure 1. Gating strategy for M1 expression in tonsillar MNC. M1 protein expression was examined in different cell populations of tonsillar MNC following MVA-NP+M1 stimulation for 18 hours. Tonsillar MNC were stained for CD19+ B cells and CD3+ T cells (a to b), followed by analysis of M1-expression in B and T cells (e). Non-B and Non-T (CD19-CD3-) cells were further separated into CD11c+ myeloid dendritic cells(mDC) and CD123+ plasmacytoid dendritic cells(pDC) (c), and cytokeratin+EpCAM+ epithelial cells (d). M1 expression in mDC, pDC and epithelial cells was analyzed and shown in f, g and h respectively.

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