DEPARTMENT OF INFECTION AND IMMUNITY

Influenza vaccinology and clinical assessment

In support of submission for the degree of Doctor of Philosophy (Ph.D.)

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1. Acknowledgements

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In Oxford, Professors Sarah Gilbert and Adrian Hill for supervising my work there, and allowing me to use that work to form the bulk of this thesis. Also in Oxford, the other clinical research fellows, Christopher Duncan, Susanne Sheehy, Joel Meyer and Ros Rowland, as well as the flu administration team of Katherine Gantlett and Laura Dinsmore, for making the job such an enjoyable and productive year. In the lab at the Jenner Institute, Tara Berthoud deserves particular thanks for attempting to show me how to do immunological experiments, and having patience when I kept getting them wrong.

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2. Abstract

Influenza virus infection is a global health problem, causing both seasonal epidemics and episodic pandemics of influenza A which are associated with significant mortality and morbidity. The development of influenza vaccines stimulating protection against both antigenically drifted seasonal virus as well as new pandemic antigenically shifted virus would be a major advance. A vaccine that stimulates cellular immunity to conserved viral antigens is a potential area of interest, and could generate heterosubtypic immunity. I have been the lead clinician for both phase I and II studies of thenovel viral vectored vaccine MVA-NP+M1 (modified vaccinia virus Ankarra, expressing nucleoprotein and matrix protein 1), designed to induce cellular immunity to influenza A virus. In this role I have been involved in the design, ethical and regulatory approval of phase I studies of safety and immunogenicity of MVA-NP+M1, as well as recruiting and vaccinating volunteers. The phase I studies showed that the vaccine was safe and immunogenic in both young and elderly volunteers. In addition to my clinical role in the phase I studies, I performed laboratory based immunological assays of immunogenicity (ELISPOT testing) in both phase I and II studies. For the phase II study, I lead the safety challenge study as well as collecting, analysing and writing up the data from the quarantine challenge study. This phase II study showed that MVA-NP+M1 is partially protective against influenza challenge in healthy volunteers, that the challenge model to assess protection is safe and that further challenge studies are warranted. I have also initiated two separate clinical studies on influenza, one comparing early clinical features of influenza with those of malaria, both from volunteer challenge studies, while the other study was of the clinical assessment of severity of influenza during a busy winter influenza season, with particular reference to those patients requiring critical care. For both studies I conceived the idea, organised the data collection and analysed the data. These data are of use in pandemic settings in allowing the assessment of patients with influenza and in determining the appropriate setting for their care.

3. List of abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AMA 1	Apical membrane antigen 1
BCG	Bacille Calmette-Guerin
CAP	Community acquired pneumonia
CD	Cluster of differentiation
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CSP	Circumsporozoite protein
CURB-65	Confusion, Urea, Respiratory rate, Blood pressure, age over 65
CXR	Chest X-Ray
DNA	Deoxyribonucleic acid
ELISPOT	Enzyme linked immunospot
FP	Fowl Pox
HA	Haemagglutinin
HI	Haemagglutinin inhibition
HIV	Human Immunodeficiency Virus
HLA	Human leucocyte antigen
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interlukin
ILI	Influenza like illness
ITU	Intensive therapy unit
LAIV	Live attenuated influenza vaccine
M1	Matrix protein 1
ME-TRAP	Multi epitope thrombospondin related adhesion protein
MEWS	Modified early warning score
MSP 1	Merozoite surface protein 1
MVA	Modified vaccinia virus Ankara
NA	Neuraminidase
NHS	National Health Service
NNT	Number needed to treat

NP	Nucleoprotein
NPV	Negative predictive value
PB 1 / 2	Polymerase basic protein 1 / 2
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPV	Positive predictive value
PSI	Pneumonia severity index
SFU	Spot forming unit
TB	Tuberculosis
TIV	Trivalent inactivated vaccine
UK	United Kingdom
USA	United States of America

4. List of papers, together with contributions to published works forming thesis

1. Berthoud TK, Hamill M, **Lillie PJ**, Hwenda L, Collins KA, Ewer KJ, et al. Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2011;52(1):1-7.

For this paper, I performed clinical follow up of vaccinated volunteers, data collection of adverse events and collection of specimens for immunological analysis. I analysed the safety data and wrote the methods section on volunteer recruitment, follow up and adverse events.

2. Antrobus RD**, Lillie PJ**, Berthoud TK, Spencer AJ, McLaren JE, Ladell K, et al. A T-cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. PloS one. 2012;7(10):e48322. Joint first authorship.

For this paper I designed the study (in conjunction with Professor Gilbert) and prepared and submitted the ethical and regulatory approvals. I recruited, screened and vaccinated volunteers, as well as following up volunteers for adverse events and collecting specimens for immunological analysis, to which I also contributed in the laboratory. I was involved in writing the sections of the paper regarding volunteer selection and adverse event analysis.

3. **Lillie PJ,** Berthoud TK, Powell TJ, Lambe T, Mullarkey C, Spencer AJ, et al. Preliminary assessment of the efficacy of a T-cell-based influenza vaccine, MVA-NP+M1, in humans. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2012;55(1):19-25.

For this paper I performed recruitment and follow up of volunteers, and was responsible for the administration and running of the influenza challenge in the pilot study. I was involved in running the quarantine challenge and collection of all specimens for

immunological and virological analysis, together with clinical assessment of volunteers and collection of clinical data. I assisted in ELISPOT testing in the laboratory, and analysed safety, immunological and clinical data. I was involved in writing the paper, in particular the sections on adverse events and clinical outcomes of the challenge study.

4. Powell TJ, Peng Y, Berthoud TK, Blais ME, **Lillie PJ**, Hill AV, et al. Examination of influenza specific T-cell responses after influenza virus challenge in individuals vaccinated with MVA-NP+M1 vaccine. PloS one. 2013;8(5):e62778.

For this paper I collected specimens, assisted in ELISPOT testing, analysed clinical and immunological data and assisted in drafting the paper.

5. **Lillie PJ**, Bazaz R, Dexter L, Biju C, Peart L, Lewis L, et al. Severity assessment of influenza virus infection in secondary care. The Journal of infection. 2012;64(2):239-41.

For this paper I had the idea for the study, collected the data, provided direct clinical care or clinical advice to the patients, analysed the data, devised the assessment protocol and wrote the paper.

6. **Lillie PJ**, Duncan CJ, Sheehy SH, Meyer J, O'Hara GA, Gilbert SC, et al. Distinguishing malaria and influenza: early clinical features in controlled human experimental infection studies. Travel medicine and infectious disease. 2012;10(4):192-6. Joint first authorship.

For this paper I conceived the idea and collected the data from the influenza section of the paper as described in paper 3. I analysed the clinical data and jointly wrote the paper.

5. Commentary

5.1 Overview of morbidity and mortality due to influenza infection

5.1.2 Mortality and morbidity prior to the emergence of the 2009 H1N1 pandemic

Influenza A virus was discovered in the early 1930s in the aftermath of the first influenza pandemic of the twentieth century (1). Influenza disease had been known for many years prior to the discovery of the causative virus and the mortality and morbidity has been well established over time. Whilst attention is drawn to the effect of pandemic influenza, such as the 1919 Spanish flu in which an estimated 40 – 50 million deaths occurred (2), the year on year mortality and morbidity associated with seasonal and local epidemic influenza infection is a substantial burden on health care systems. A retrospective cohort study from the USA showed that in a non-epidemic influenza season (1970-1971) the rates of pneumonia and influenza related death and hospitalization were 6 / 100,000 and 58 / 100,000 respectively (3). In the epidemic years studied, the rates of hospitalizations, influenza and pneumonia mortality as well as all-cause mortality were significantly higher, with 11-13 excess deaths / 100,000 population, with a 140% increase in the hospitalisation rate, and an increase in the case fatality rate from 9.5% to 12-13%. This pattern of excess deaths occurring in influenza seasons has been shown in other studies. A retrospective mathematical modelling study of excess deaths attributable to influenza in England and Wales, between the 1999/2000 and the 2009/2010, showed up that in seasons of low activity there were just over 7000 influenza related deaths, whilst in the 1999/2000 influenza season (a period of high activity) there were just under 25,000 deaths related to influenza infection (4). A modelling study using data from the USA, on influenza seasons from 1972 – 1992, showed that on average there were an excess of 21,300 deaths per season (5).

In addition to the excess mortality, influenza leads to increased healthcare utilization and economic loss through time of work and reduced productivity. In a cohort study in Michigan, depending on age and the prevalent circulating influenza strain, between 110 –

1023 bed or restricted activity days / 1000 people / year occurred (6). In a UK study of a single company, there was a mean of 2.8 days away from work due to an influenza like illness (ILI) (7), with reduced productivity noted for those who continued to work despite being symptomatic from their ILI, despite high levels of use of medications for symptomatic relief. An estimate of the economic cost of influenza infections in the USA is that direct medical costs of seasonal influenza were $10.4 billion, with total economic costs being $87.1 billion (8). At the more severe end of the spectrum, hospitalization is also seen to increase due to influenza infection. A study from the Netherlands showed that during the influenza seasons of 1996 – 2000, hospitalizations for respiratory complications , cardiovascular disease and pneumonia and influenza were significantly increased, and that this was more marked in the elderly and those with co-morbidity (9). In the study by Molinari, over 330,000 patients were hospitalized, for 3.1 million days (8).

Whilst influenza infection can affect people of any age, the burden of severe disease is carried predominately by the elderly, the very young and those with co-morbid conditions. A three year survey of respiratory hospitalizations in Houston, showed that both during epidemic and non-epidemic influenza seasons, children under four years old and adults over 65 accounted for the majority of hospital admissions due to influenza related respiratory disease (10). In terms of mortality, older adults account for the greatest proportion of influenza related deaths, with a modelling study of mortality across the USA related to influenza between 1972 and 1985, estimating that influenza associated pneumonia mortality was up to 104 times higher in those over 64 years old, compared to the younger age group (11). Using national surveillance data in the United States, one study has shown that the mortality associated with influenza infection was mostly in those aged over 65, and was in large part due to death from cardio-respiratory disease as opposed to influenza and pneumonia (12). In a study of nursing home residents, both those with and without co-morbid conditions had high rates of antibiotic use for respiratory disease, hospitalization and death, with 3.4 – 3.8% of deaths attributable to influenza (13). A cohort study from Tennessee of patients with chronic lung conditions, showed that in both young children and adults over 65 years old, hospitalizations were significantly increased with influenza infection, with influenza accounting for up to 3% of all deaths in this group (14). The same group have also looked at the association between invasive pneumococcal disease (which affects the elderly and those with chronic lung conditions to a greater extent) and influenza infection (15). This study showed that the peak of pneumococcal disease was associated with both current influenza infection and

prior influenza infection, with the peak of influenza associated pneumococcal infection occurring up to four weeks post influenza infection. The effect on elderly patients is not limited to mortality or hospital admission. One study showed that frail elderly patients in residential care homes where there were outbreaks of influenza, had significant impairment of functional status up to four months post infection (16).

In addition to the elderly, those with certain co-morbid conditions are at higher risk of mortality and morbidity associated with influenza. Chronic respiratory conditions, such as chronic obstructive pulmonary disease (COPD) and asthma have been shown to be associated with higher rates of hospital admission, especially in low income groups (17). In addition to those with chronic respiratory disease, other co-morbid conditions can be exacerbated by influenza infection (9). This finding was substantiated by a study that showed influenza vaccination reduced the rates of hospital admission for cardiac disease, stroke, pneumonia or influenza, as well as all-cause mortality in two consecutive influenza seasons, in elderly patients (18). Other at risk groups, who tend to have a younger age profile than those commonly thought to be at risk, that have been shown to have poorer outcomes following influenza infection include those with HIV infection and pneumonia (19), and pregnant women (20).

5.1.3. Mortality and morbidity since the 2009 H1N1 influenza pandemic

The first influenza pandemic of the 21st century began in North America in 2009 (21, 22). An increase in influenza cases, together with a marked change in the age distribution of severe cases, with a striking increase in young adults suffering from severe disease was noted. A swine origin influenza A virus was found to be the causative virus, and studies using sera of a variety of age groups found that those born before 1920 had higher levels of neutralising antibody, suggesting that the swine origin 2009 H1N1 virus was antigenically similar to the 1918 H1N1 virus (23). The spread of the virus, together with the shift in those affected signalled the arrival of the pandemic. During the first wave of cases in early 2009, the demographic characteristics of severe cases continued to show that children and young adults, including pregnant women, were prominently represented compared to severe cases of seasonal influenza infection(24). In the UK, the initial wave of cases again showed a predilection for younger age groups, but with no mortality noted in the first reported cases (25). Whilst the first wave in the UK may have had little impact

with regard to mortality, the first major influenza season, in the southern hemisphere, after the pandemic was declared, had a major impact on health care services and a noticeable mortality (26). The critical care services in Australia and New Zealand noted several features that were to become common in the subsequent waves of the pandemic, notably the effect of obesity on outcome, the young age of severe cases and the effect of pregnancy on the course of infection (26, 27). During the first waves of influenza infection after the emergence of pandemic H1N1, the majority of illness and infection was related to the pandemic strain, with a cross sectional serological study in UK estimating that up to 42% of children under 15 years of age may have been infected during the initial period of the pandemic (28). In subsequent influenza seasons, whilst pandemic H1N1 continued to be a major circulating strain, influenza B virus co-circulated widely (29) and was responsible for severe illness (30). In addition to influenza B virus, one group showed three separate influenza A virus co-circulated, and that of the three circulating influenza A viruses (pandemic H1N1, H3N2 and seasonal H1N1), there is little difference in clinical presentation (31). Moreover a group from Hong Kong showed that pre-pandemic strains, including influenza B, all caused severe illness and mortality (32).

The first full influenza season in the UK after the pandemic in 2010/2011, was a period of high influenza activity, both of pandemic H1N1 and influenza B (29). Whilst the first wave of pandemic cases were restricted to certain geographical areas (London and the West Midlands in particular) during the 2010/2011 season the whole of the country experienced high levels of influenza activity, with critical care admissions for influenza related illness being fourfold higher than the preceding season (29). The majority of the severe cases continued to be in young adults, as was the majority of reported mortality directly from influenza. A study from the Netherlands, comparing the impact of seasonal and pandemic influenza, found that due to the lower rates of illness in the elderly with pandemic influenza, observed total mortality was lower during the pandemic, with only young children showing an increased mortality compared to seasonal infection (33). Even allowing for the likely lower mortality in older patients, a modelling study using attack and mortality rates from 12 different countries estimated that there had been 201,200 respiratory deaths in the first 12 months of pandemic H1N1 transmission, with another 83,300 cardiovascular deaths also attributable to the pandemic (34).

5.2 Immunity to influenza

5.2.1 Humoral and mucosal immunity

Given the extent of the morbidity and mortality associated with both seasonal and pandemic influenza infection, protection from influenza infection (and potentially a reduction in severity in those who are infected) is an area of great interest. Early studies of vaccination with inactivated virus showed that induction of antibodies could be protective, but that this protection was not cross specific between influenza A and B (35, 36). A later study found that the specificity of anti-influenza antibodies was critical to protection (37), and that this may be the reason for the lack of cross protection after infection or vaccination with one specific sub type. The two surface glycoproteins of influenza viruses, haemagglutinin and neuraminidase, both generate an antibody response. The response to the haemagglutinin, measured by haemagglutination inhibition (HI) assay, is considered to be the major protective response against influenza infection. In a human volunteer challenge study, the levels of HI antibody were correlated with protection from infection (38), with the protective dose for 50% (PD50) being a titre between 1:18-1:36. This study, together with others that showed similar results (39), have guided the use of HI titres to determine influenza vaccine efficacy (40), with a titre of 1:40 being labelled as seroprotective. A mismatch between vaccine strains and the circulating influenza viruses can lead to a poor serological response (41), particularly in the elderly and may be related to reduced clinical efficacy. Of note is an experimental study that showed CD4 T-cells could help antibody production against haemagglutinin, even when the T-cell was specific for a different internal influenza antigen (matrix protein) (42). A recent study using a microneutralisation assay to study cross reactive haemagglutinin antibodies to the 2009 pandemic H1N1 virus (43), showed that in children, recent vaccination with seasonal influenza vaccine (either live attenuated or inactivated) did not increase antibody to pandemic H1N1. In young adults in the year before the pandemic, only 6% before and 7% after seasonal influenza vaccination, had protective antibody titres against pandemic H1N1 (43). Interestingly, older adults had higher levels of cross protective antibody, as did those who were vaccinated with the monovalent swine flu vaccine in 1976 (43).

Whilst the levels of HI antibody are important in preventing infection, other serological responses may play a part in either preventing or ameliorating the clinical course of infection. Serum levels of anti-neuraminidase antibodies reduced both viral shedding from the upper respiratory tract, as well as decreasing the severity of clinical illness in experimentally infected volunteers (44). In addition to serum antibody, the role of nasal antibodies and cytokines, has been studied, both free(45) and epithelial cell bound(46). The study by Clements et al (45), showed that a variety of both serum and nasal antibodies had effects on viral replication and illness severity, with anti-neuraminidase antibody having the broadest range of effects. However, the challenge virus was homologus to the vaccine and therefore little can be said about cross protective effects of different antibodies. Nasal cytokine production, in response to virus challenge has also been shown to have an effect on symptoms and viral shedding (47, 48). Whilst these data show that cytokines and chemokines have an effect on symptoms and viral shedding, they do not prevent viral infection and may contribute adversely to symptoms and clinical illness.

5.2.2 Vaccine induced immunity and its clinical efficacy

Currently influenza vaccination is recommended for those aged 65 and over, as well as patients with chronic health conditions that put them at increased risk of severe illness from influenza. However the efficacy of vaccination is a much debated area, with one meta-analysis and review estimating that vaccine efficacy for preventing laboratory confirmed influenza is 63%, but that this decreases markedly in the elderly and that protection against clinical influenza is lower at 22%(49). A more recent and strict meta-analysis, looking only at laboratory confirmed influenza gave a median vaccine efficacy for trivalent influenza vaccine (TIV) of 62%, whilst live attenuated influenza vaccine (LAIV) had a median vaccine efficacy of 78% (50). However none of the trials of TIV in the later meta-analysis included adults aged 65 or more, who are obviously one of the main target groups for influenza vaccination. One placebo controlled trial looking specifically at TIV effectiveness against both clinical and serologically confirmed influenza in patients over 60 years of age (51), found a 50% reduction in serological and a 47% reduction in clinical influenza respectively. The relative efficacy of LAIV and TIV has been addressed in two separate randomised trials (52, 53) which suggest that LAIV has greater efficacy at preventing culture confirmed influenza in children (52), whilst TIV appeared to have

greater efficacy at preventing PCR and culture confirmed influenza in young adults (53). As LAIV appears to have a priming effect on T-cell immunity against influenza in children but not in adults (54), this may be one of the contributing factors to this difference in efficacy.

Several cohort studies and clinical trials that did not report laboratory confirmed influenza as a primary outcome have shown effectiveness of influenza vaccine in reducing a variety of clinical outcomes. Two studies using the UK general practice research database (55, 56), have looked at the effect of influenza vaccination on all cause, as well as respiratory and cardiac mortality. In the prospective cohort study described by Armstrong *et al* (55), mortality was higher in non-vaccinated patients during periods of high influenza activity, whilst for vaccinated patients mortality rates did not significantly increase at these times. This was true for all cause and respiratory mortality (55). The retrospective cohort study that was conducted by the same group (56) found that seasonal influenza vaccination was 21% effective in reducing hospital admission for respiratory illness over the nine year period of the study, with a 12 % reduction in all-cause mortality. The effectiveness of the vaccine seemed to be related to the severity of the influenza season, with higher efficacy seen during the most severe influenza seasons (56). Other clinical outcomes that have been assessed in cohort studies have included the effect of influenza vaccine on pneumonia mortality and outcomes during four consecutive influenza seasons (57). Current (that season's vaccine) vaccination was associated with reduced in hospital mortality, which was consistent after correction those with unknown vaccination status. It also found that this effect was consistent in both younger and older patients, as well as those with severe community acquire pneumonia (CAP) (57). Interestingly, the effectiveness of vaccination was greatest in those seasons when vaccine strains were most closely matched to the circulating strains. In addition to this potential effect on outcomes in patients with CAP, another study focused on the potential effect on vascular outcomes in older patients who received influenza vaccination (18). The findings from this large cohort study (over 280,000 subjects across two influenza seasons) were that vaccination against influenza, in a population with a range of co-morbid conditions, reduced not only the rate of cardiac or cerebrovascular hospital admissions, but also reduced all-cause mortality, with a number needed to treat (NNT) to prevent death or hospitalisation of 61 in the 1998-1999 influenza season, and an NNT of 68 in the 1999-2000 season (18).

5.2.3 Cellular and heterosubtypic immunity to influenza A

Whilst antibody mediated immunity to surface glycoproteins may generate protective immunity, the nature of antigenic variation in these molecules, both in terms of seasonal antigenic drift and emergence of antigenically shifted pandemic virus, means that cross specific immunity (heterosubtypic) is unlikely to be successful. A study conducted early in the emergence of the 2009 pandemic showed that B cell / antibody epitopes were poorly cross reactive between pandemic H1N1 and the previously circulating H1N1 strains (58). This study did show that there were a much greater number of conserved epitopes recognised by T lymphocytes, and that this was split between both CD8 and CD4 T-cells (58). The nature of the T-cell response and the potential for it to be induced by vaccination are the main areas that will be covered in the majority of this thesis.

The effect of cellular immunity in curtailing influenza infection is most clearly shown in challenge studies conducted on volunteers with no detectable humoral immunity to influenza. A key study in this regard is the paper from McMichael's group in 1983 (59). Whilst this study confirms the importance of HI titres in preventing infection (no volunteer with a titre of >1:20 shed virus), the results showed that in the presence of influenza virus, T-cells could recognise and lyse infected cells (as measured by a chromium release assay) clearing viral infection, even in those without pre-existing antibody being present. This study also provided evidence that cross sub type immunity might be related to cellular immunity, as volunteers who were born after 1956 were exposed to virus that was not circulating during their lifetime, still cleared the infection in the absence of any humoral response (59). The potential for T-cells to recognise epitopes that are present in different sub types of influenza A has been further investigated, with the nature of the cross recognition being elucidated. Using enzyme linked immuno spot (ELISPOT) techniques, T-cells (both CD4 and CD8) from British volunteers were shown to recognise epitopes from both seasonal H3N2 influenza A and avian H5N1 which causes sporadic severe disease in South East Asia and is considered to have pandemic potential (60). The targets for cross recognition were explored using the whole proteome of both H3N2 and H5N1 viruses, with the matrix protein 1 (M1) and nucleoprotein (NP) being the major targets recognised in both viruses (60). A subsequent study showed that cytotoxic T-cells generated by exposure to seasonal influenza viruses (both H1N1 and H3N2), could recognise and lyse cells infected with pandemic H1N1 (61). Using HLA tetramer staining techniques, this group also showed that an epitope from M1 that was present in seasonal influenza was

also present in pandemic H1N1 and was recognised by cytotoxic cells that had not been previously exposed to pandemic H1N1 virus (61). Interestingly, a separate study looking at CD8 T-cell responses to epitopes that aren't conserved across all influenza A sub types (62), found that an epitope of the NP that was present in the 1918 H1N1, and recognised by CD8 T-cells, was also present in the 2009 pandemic H1N1.

The nature of a potentially protective T-cell response to influenza, especially with regard to antigen selection, is important in influencing vaccine strategies that could stimulate T-cell immunity. HLA restriction of the T-cell response was shown in several studies (63-66), both to CD8 (63, 64) and CD4 cells (65). These studies showed that T lymphocytes capable of lysing influenza infected cells recognised viral molecules on the surface of the infected cell. The nature of the epitopes presented in this manner was further elucidated in a series of studies by McMichael. In one of the earlier studies, T-cells were identified as the cytotoxic cell, and were shown to be able to recognise subtypes of influenza A but not influenza B (66). Further studies clarified the nature of the antigen presented to the T-cells, with evidence that both NP (67-69) and matrix protein (42, 63, 70, 71) and polymerase PB2 (71) contribute peptide molecules that are presented to HLA restricted cytotoxic T-cells. An earlier study had shown that a variety of viral proteins were presented at the cell surface and recognised by T-cells (72), and that some of these T-cells were able to recognise cells infected with any influenza A sub type.

The finding that these internal proteins are presented to cytotoxic cells and are conserved between subtypes of influenza A, together with the finding that NP and M1 are the dominant epitopes conserved between sub types (60, 61), suggests that these proteins may be suitable vaccine antigens to stimulate T-cell mediated immunity. T-cell immunity could be relevant in heterosubtypic immunity as was shown in the pivotal McMichael paper from 1983 (59). Further circumstantial evidence for the contribution of non-humoral immunity to heterosubtypic immunity is from a historical review of cases occurring during the H2N2 pandemic of 1957 (73). This study found that adults who had previously been exposed and infected with H1N1 virus prior to the emergence of the H2N2 pandemic, had lower attack rates from the pandemic H2N2 virus than children who had had no previous exposure to either virus, or adults who had not been previously infected with H1N1. Given that both the haemagglutinin and neuraminidase proteins shifted from H1N1 to H2N2 in this time period, it is unlikely that cross reactive protective antibodies to HA or NP were responsible for the majority of the effect detected, perhaps suggesting that cell mediated immunity played a significant role. T-cell cytotoxic capability has been

shown to be “boosted” by exposure to natural influenza infection (74) with a half-life of the cytotoxic cell of two to three years and noticeable waning of T-cell cytotoxic capability, indicating that there is the potential for vaccination to increase this element of influenza immunity.

Some earlier studies have shown that live attenuated influenza vaccine can prime T-cell responses in children without detectable pre-existing T-cell responses, but do not boost those responses in adults (54). Inactivated current TIV, in particular the split virion type, can stimulate modest T-cell responses (75), although the protective effect of this effect is likely to be moderate. One study has shown that there is a small amount of M1 and NP in trivalent vaccine and that in HLA-A2 positive individuals this can stimulate cellular responses and cytotoxicity *in vitro* (76). In older vaccine recipients, the effect appears to be lower than younger patients (77), but by assessing cellular responses, particularly granzyme B responses (78, 79) it may be possible to assess those elderly patients at increased risk despite vaccination. A study looking at the relationship between T-cell subsets and HI titres after vaccination with trivalent inactivated influenza vaccine, showed that higher levels of $CD8^{+}$ $CD28^{null}$ T-cells, were associated with poor vaccine responses, as defined by reduced antibody titres to vaccine strains (80). Children vaccinated with the 2009 pandemic H1N1 monovalent vaccine have been shown to have a small but measurable increase in T-cell responses to internal antigens when subsequently vaccinated with TIV (81).

One of the major problems in assessing the effectiveness of T-cell immune protection (not just in influenza) is the lack of good correlates of protective immunity. Two recent studies, one a human volunteer challenge study (82), the other a longitudinal cohort study (83), have shed new light on the nature of cellular immune protective correlates against influenza. The challenge study described the effect of pre-existing T-cell responses on viral shedding and the clinical course of volunteers nasally inoculated with either H3N2 or pre pandemic H1N1 virus. Pre-existing responses were predominately CD4 in nature, with NP and M1 being major targeted proteins. Cytotoxic CD4 T-cell responses against influenza peptides appeared to be correlated with reduced viral shedding and lower symptom scores, but there was little contribution of CD8 T-cells directed against influenza derived peptides, despite evidence of both CD4 and CD8 T-cells exhibiting cytotoxicity via the perforin-granzyme pathway (82). As CD8 cells were found in lower numbers pre-challenge in this study, it might be that at baseline levels they play less of a role in limiting influenza infection, but the cohort study described by Sridhar and colleagues (83) conducted during

the 2009 pandemic indicated that anti influenza CD8 cells of a specific phenotype ($CD8^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}$) were associated with reduced incidence of influenza like illness, reduction in viral shedding and lower symptoms scores. Further specific study of the protective nature of these CD8 cells showed they recognised conserved epitopes of PB1, M1 and NP, and had cytotoxic and lung homing capabilities (83). Thus it appears that cytotoxic CD4 and CD8 T-cell responses may both contribute to antiviral responses against influenza.

5.3 Viral vectored vaccines to stimulate cellular immunity

As cellular immunity appears to have a potential bearing on influenza infection, and could offer heterotypic protection, development of a vaccine that boosts this component of the immune response is an attractive option. Viral vectored vaccines have been developed and shown to be potent at stimulating immune responses to a variety of antigens and this section of thesis will consider the development of these vectors.

Whilst many infections can be targeted with antibody producing vaccines, intra-cellular organisms such as tuberculosis or those with complex infective life cycles such as malaria may not be as susceptible to humoral mediated immune clearance. By identifying epitopes that are recognised by T-cells, and inducing cellular responses to these targets, intra-cellular pathogens, and some cancers, may be susceptible to immune clearance mediated via T-cells.

One of the most studied viral vectors capable of inducing cellular immune responses is the modified vaccinia virus Ankara (MVA) (84-86). This virus was originally used as a smallpox vaccine in Germany and Turkey and was safely given to over 120,000 people (84). It is unable to proliferate in human cells, and has its genome shortened by approximately 9% compared to reference strains of vaccinia virus (84). However, despite not being able to replicate in humans, viral DNA is processed and viral protein assembly occurs, being incomplete only at a late stage of assembly (85, 86). Crucially it is also able to express DNA that has been cloned and inserted into its genome (85). It is therefore able to express sequences of DNA that code for antigens of interest, which can then be processed and presented at the cell surface for immune recognition. MVA, when used as a smallpox vaccine, was administered by intradermal, subcutaneous and intramuscular routes, and was given to a wide age range (84, 86), without major adverse effects. It also appears to

induce interferon production, potentially assisting the immune response to the inserted antigen (86).

Clinical studies of MVA, expressing a variety of target antigens have taken place in many patient groups and geographical settings. MVA has predominately been used to boost T-cell responses that have been primed by another route, the "heterologous prime boost" approach (87). Table 1 lists some of the clinical studies in which MVA has been used in this method. Of importance is the study by Vuloa *et al* (88). This study looked at the immunogenicity of different combinations of vectors in a heterologous prime boost strategy for the malaria antigen, multi epitope thrombospondin related adhesion protein (ME-TRAP). The vectors used were plasmid DNA, MVA and attenuated fowlpox 9 (FP9) virus. The greatest immunogenicity (both in terms of the magnitude of the T-cell ELISPOT response and its longevity), were observed when MVA was the vector used to boost a prime from the other vectors (88). Importantly, however, this approach had not previously been employed as a vaccine strategy, in humans, against influenza A virus and the papers which form this thesis form the first published reports of this strategy.

Disease	MVA antigen	Priming method	References
Malaria	ME-TRAP, CS, AMA1, MSP1	DNA, Adenovirus, fowlpox virus	(88-93)
Tuberculosis	85A	BCG, latent TB infection	(94-97)
HIV	nef, gag / multi epitope	HIV infection	(98, 99)
Influenza	NP+M1	Past infection, Adenovirus	(100-103)
Melanoma	Human tyrosinase, multi epitope	Melanoma	(104, 105)
Renal cell cancer	5T4	Renal cancer	(106)

Table 1 – Clinical studies of MVA as a vaccine vector. Abbreviations: AMA1, Apical Membrane Antigen 1; BCG, Bacillus Calmette-Guerin; CS, Circumsporozoite protein; ME-TRAP, Multi Epitope Thrombospondin Related Adhesion Protein; MSP 1, Merozoite Surface Protein 1; NP+M1, Nucleoprotein and Matrix protein 1.

The safety profile of MVA as a vector (as opposed to its use as a smallpox vaccine), has been assessed in all of these studies, which have included children and infants (90, 97), elderly patients (101, 106), HIV-1 seropositive individuals (98, 99), as well as African volunteers (89, 97). In a detailed report on the safety of both MVA and fowlpox vectors (91), local reactions (to intradermal administration of vaccine), were the most common adverse event, with systemic adverse events generally being mild and short lived. By comparing different routes of administration (intradermal and intramuscular), and different doses, the first of the papers forming the basis of this thesis (100) gives further insight into the side effect profile of MVA vectors. As might be expected, administration as an intradermal injection led to greater erythema, itch and swelling, whilst systemic symptoms were related to the vaccine dose, with the high dose group suffering more severe adverse events, in particular fever and malaise (100). It is also of note that adverse events, when using the same dose and route of MVA-NP+M1, were similar in both young and older volunteers (101, 102).

The potential for the use of MVA based vaccines to induce T-cell based heterosubtypic immunity is the basis for papers 1 – 4 in this thesis (100-102, 107). The aim of the research was to investigate if by boosting pre-existing T-cell responses, which are conserved across multiple influenza A sub types, clinical disease could be reduced. The phase I vaccine studies (100, 101) concentrated on demonstrating safety and immunogenicity of the candidate vaccine, MVA-NP+M1. The first trial was in younger adult volunteers and showed that the vaccine was safe via both intramuscular and intradermal routes, and was able to boost pre-existing T-cell responses, in both CD8 and CD4 cells (100). Given the findings of the studies looking at correlates of cellular immunity (82, 83) the ability to generate both a CD4 and CD8 response would seem to be of benefit. The second trial assessed the immunogenicity and safety in an older group of volunteers, who by virtue of their age would be at greater risk from influenza infection (101). In this group there continued to be a significant increase in both CD4 and CD8 T-cells recognising the vaccine antigens, and a similar level of functionality of these cells was seen as in the study of younger volunteers (100). The safety data in this elderly group of volunteers was the same as observed in the younger volunteers vaccinated with the same dose. A previous study had found that a high IFN-γ:IL-10 ratio was associated with protection from influenza in the elderly (78), and the increase in IFN-γ secretion seen after vaccination with MVA-NP+M1 was not associated with an increase in IL-10 levels, which may contribute to increased protection in the elderly (101).

The next clinical trial of MVA-NP+M1 involved a human challenge study (102), together with further immunological studies of vaccinated volunteers (107). The timeline for the challenge study is shown in Figure 1.

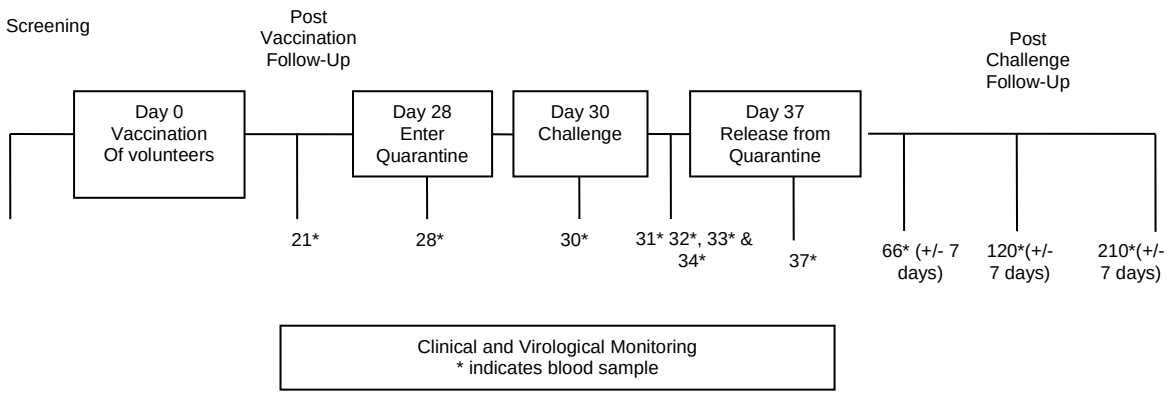

Figure 1 – Timeline of the influenza challenge study in reference 65.

In this clinical trial, the trend across all clinically observed measures was of MVA-NP+M1 being beneficial, in addition to showing that the challenge model after vaccination was a safe mode of assessing efficacy when using a novel T-cell stimulating vaccine. The immunological studies showed that vaccinated volunteers had higher levels of anti viral T-cell cytolytic activity and T-cell activation after influenza challenge (107), which might contribute to enhanced viral clearance and attenuation of symptom, however in this clinical study no immunological outcome was of use in predicting protection from influenza disease or viral shedding. These same immunological correlates had in an earlier study been shown to be a better correlate of protection from influenza than HI titres (78). The research in papers 1 – 3 also adds to the safety data around the use of MVA as a viral vector and extends the safe use of this vector to an older cohort. The challenge study performed as part of paper 3 (102) also showed that this method of testing a novel T-cell stimulating vaccine was safe, and potentially useful in exploring vaccine effectiveness.

5.4 Human challenge studies of influenza infection

The use of human volunteers as a model in infection research has many advantages. By studying the human response to infection, no extrapolation from animal studies is needed, and the effect of vaccines, drug treatments and the natural history of the infection can be ascertained. Safety concerns are of paramount importance, particularly when potentially fatal infections such as malaria are given to volunteers. A group of UK and American researchers have put forward the view that human microbial challenge is the “ultimate animal model” (108). Within influenza research, the use of challenge studies has allowed assessment of the natural time course and severity of infection (109), which, as the point in time of naturally acquiring influenza is not often known, is of great use. This review of influenza challenge studies has confirmed the relationship between the amount of virus shed and clinical symptoms, whilst also showing that asymptomatic viral shedding does occur. It has also highlighted that viral shedding peaks early after inoculation and that this may limit the ability to contain the spread of infection (109). Other influenza challenge studies have helped to elucidate the mechanisms of cellular immunity to influenza (59, 82), the efficacy of neuraminidase inhibitors in limiting symptom duration (48, 110), the

local and systemic cytokine and chemokine responses to infection and their association with symptoms (47, 48), the role of mucosal antibody in protection from infection (45), gene expression profiles that may differentiate viral from bacterial infection (111) and the efficacy of vaccines (102). Perhaps one of the major factors limiting the generalizability of influenza challenge studies is the route via which volunteers are inoculated. In the majority of studies nasal instillation of virus is used, as this allows rapid and reliable infection to occur (109). However this may not mimic the natural acquisition of influenza from aerosol transmission, and further challenge studies have been planned to look at the efficacy of protective equipment in preventing infection via the aerosol route (112).

5.5 Clinical assessment of influenza illness

5.5.1. Severity assessment of influenza infection

Influenza infection can present in a variety of ways, and the spectrum of severity, can run from a symptomatic infection (28, 109) through to fatal severe illness (32, 113). During periods of high influenza activity critical care services are under extreme pressure (26, 29) and effective early triage of those patients who require intensive care support would be advantageous. Conversely identifying those patients at low risk for severe disease may allow that group to be managed in the community freeing up capacity in secondary care for the more severely ill (114). Paper 5 of this thesis (30) relates to the assessment of severity of influenza infection , and to the development of a proposed triage tool, during an influenza season with a large number of cases managed in secondary care at Sheffield Teaching Hospitals NHS Foundation Trust. Figure 2 shows the time frame of the cases over the winter of 2010 / 2011.

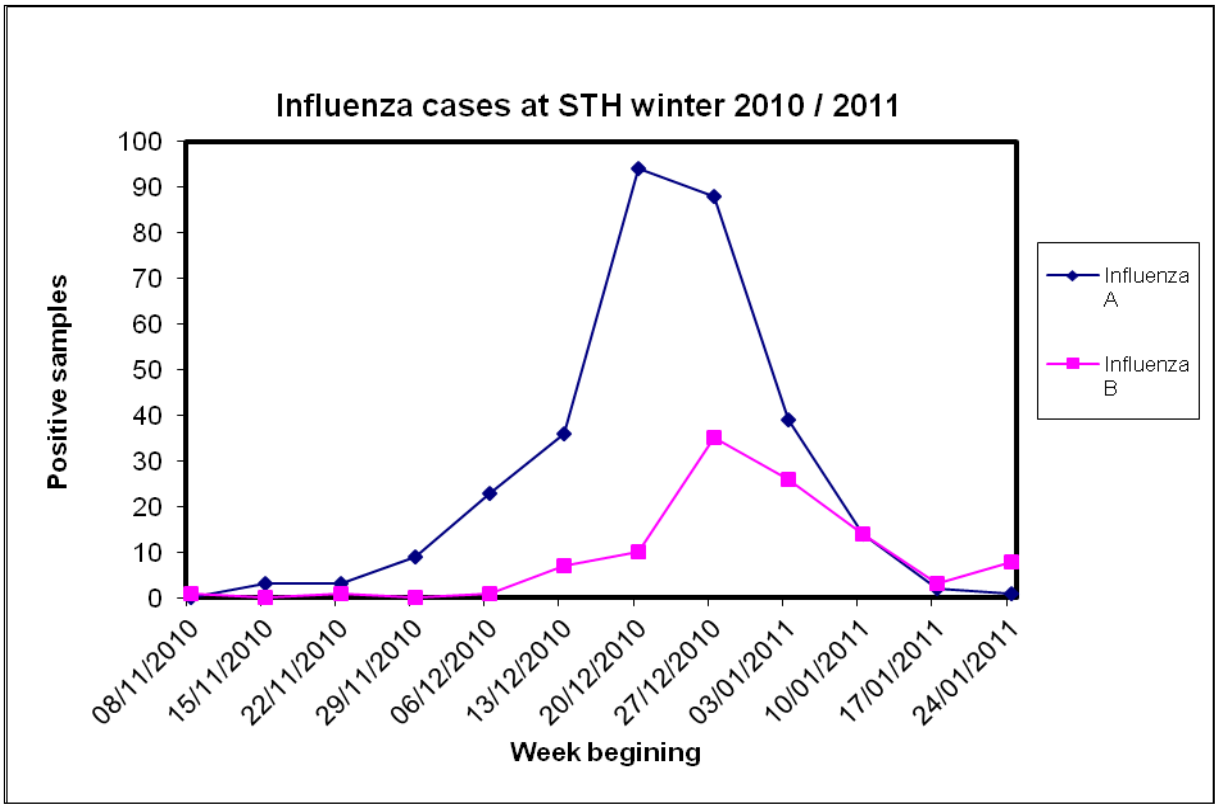

Figure 2 – Time course of influenza cases at Sheffield Teaching Hospitals Winter 2010 / 2011.

Whilst there is an extensive literature on severity assessment in community acquired pneumonia (CAP), with validated assessment tools such as the pneumonia severity index (PSI) (115), CURB-65 (116) and SMART-COP (117), these may not be as readily applicable to influenza infection. A study from Canada looked at the use of pneumonia assessment tools for predicting outcome in influenza infected patients (118). In this study, over three influenza seasons (all before the emergence of pandemic H1N1), patients admitted to hospital who had a positive sample for influenza had eight separate severity scores calculated (both pneumonia specific and sepsis scores), and rates of death and intensive care admissions were recorded. Whilst the PSI predicted mortality with reasonable accuracy, it was less effective in predicting intensive care admission and the study did not assess the utility of the scoring systems in guiding outpatient treatment (118). Another issue with this study is that since the emergence of pandemic H1N1, the average age of patients requiring intensive care with influenza infection is much younger than the average age in the Canadian study which was 76 years of age (118). A study from South Korea , in the early stages of the 2009 H1N1 pandemic found that in univariate analysis both PSI and

CURB-65 scores predicted mortality, with PSI remaining predictive in multivariate analysis (119). However one of the issues with the PSI is that it is a reasonably complex tool to use, requiring multiple steps before assigning a patient to a PSI level, and another study in CAP patients advocated using CURB-65 due to its ease of use and that the performance characteristics were not substantially different from PSI (120).

Generic severity assessment scores, such as the modified early warning score (MEWS) (121) may be of use in prognostic assessment of infection. A study comparing early warning scores, sepsis scoring and CURB-65 in CAP, found that the disease specific CURB-65 score outperformed the more generic scoring systems (122), in predicting mortality. In common with many of the studies in this area, mortality as opposed to critical care admission was the end point for this study and therefore it may be that it has less utility in predicting the need for critical care, which in influenza epidemic conditions may well be a more relevant measure.

Another caveat of studies of influenza severity assessment, as highlighted above, is that those conducted before the emergence of pandemic H1N1 may underestimate the severity in younger patients, and pregnant women. In one of the largest pre 2009 pandemic observational studies looking at risk factors for death and poor outcome from influenza infection, carried out over two years in Hong Kong, the average age of admitted patients was 70 (32), as compared with 23 years of age in the first wave of pandemic cases in the UK (123). In addition to older age as a predictor of mortality, major co-morbidity, need for ventilatory support, male sex and being a nursing home resident were associated with death (32), whilst treatment with oseltamivir appeared to be beneficial in reducing risk of death. Again, this study looked at death as its primary outcome and may not therefore be relevant when applied to decisions regarding critical care requirement. Another study from Taiwan of patients with confirmed influenza pneumonia (124), found that the strongest predictor of mortality was the degree of hypoxia, as measured by the ratio of the arterial partial pressure of oxygen to inspired oxygen concentration. As this study was in confirmed influenza pneumonia, with an overall mortality rate of 44.7%, the generalizability to non-pneumonic influenza infection in a triage setting may be limited. As both of these studies were carried out before the emergence of pandemic H1N1, and the description during the pandemic and early post pandemic period of novel risk factors such as obesity, it is unclear if those risks also pertained to other influenza virus infections.

Many studies were performed to look at the risk factors for poor outcome during the initial period of the 2009 pandemic. A UK based study investigated risk factors for death or need for critical care support during the first wave of the 2009 pandemic (123). In keeping with pre pandemic findings, the presence of respiratory co-morbidity was associated with poor outcome. One of the new factors that appeared to influence outcome in several of the studies of pandemic H1N1 was obesity (26, 123, 125), whilst pregnancy, which had been noted as a risk factors prior to the pandemic became a much more widely noted risk factor (20, 26, 27, 126). A study from early in the pandemic from Mexico reported factors that were associated with lower risk and the ability to be treated as an outpatient (127). Although mostly looking at factors associated with death, a lower respiratory rate, absence of cyanosis and not being confined to bed were associated with the ability to be treated as an outpatient (127), but these vague descriptive factors do not lend themselves to being implemented as algorithms or protocols to decide who could be treated as an outpatient. The other reports from the pandemic concentrated on risk factors for mortality or poor outcome and Table 2 shows the risk factors identified by these studies, associated with poor outcome.

Factors associated with poor outcome	**Reference**
Pregnancy	(24, 26, 27, 126)
Obesity	(26, 113, 123, 128)
Older age	(24, 26, 129)
Hypoxia / raised respiratory rate	(24, 26, 127, 130, 131)
Co-morbidities	(24, 26, 123, 127-129)
Hypotension / shock	(130, 131)
Confusion /altered mental state	(129, 130)
Delayed receipt of anti-viral treatment	(24, 126, 128, 132, 133)
Radiologically confirmed pneumonia	(24, 123, 129)

Table 2 – Factors associated with poor outcome during the initial phase of the 2009 H1N1 pandemic.

As these studies were carried out during the first waves of the 2009 pandemic, it is not surprising that they report outcomes only of patients infected with pandemic H1N1. How this can be extrapolated to influenza seasons where there is co-circulation of other influenza viruses is still unclear. One study looked at the clinical presentation and 30 day outcome of both pre and post pandemic strains of H1N1 as well as H3N2 virus (31). This study showed that pneumonia and acute otitis media were more common, in adults, with pandemic H1N1 than either H3N2 or pre pandemic seasonal H1N1 (31). However this study did not collect data on risk factors for complications or poor outcome, and did not look at outcomes with influenza B virus infection. What the study described in paper 5 (30) adds to the literature is detail on the factors associated with the need for critical care during both pandemic H1N1 and influenza B infection, in the early post pandemic period. It also addresses potential factors associated with admission for less than 24 hours, which may allow early discharge or community based treatment with anti-viral medication. In one retrospective Chinese study, outpatient use of oseltamivir reduced the risk of progression to severe disease and pneumonia (134), and it may therefore be appropriate that those in lower risk groups for complications are not admitted to hospital, but do still receive antiviral treatment to reduce the risk of complications. The classification into groups that are suitable for outpatient therapy and those that may require critical care, in a simple manner, is one of the major uses of severity assessment in CAP. Paper 5 proposes a two-step assessment process, firstly to assess whether the patient is suitable for outpatient treatment, and then if not appropriate for outpatient management, an assessment of the need for critical care is carried out. This contrasts with the other published studies which concentrate on risk assessment for poor outcomes only.

5.5.2 Distinguishing influenza and malaria clinically

At the beginning of the 2009 pandemic in the UK, a national telephone triage service was implemented to assist in the allocation of anti-viral medication (135), which used the HPA case definition for pandemic influenza (136). Soon after this there were reports of misdiagnosis of other severe illness as “swine flu” after use of the telephone triage system (137-139). Subsequently reports from the UK and Korea questioned the utility of case definitions in pandemic influenza (140, 141), particularly with regard to the sensitivity to

predict influenza, and a meta-analysis of decision rules for influenza infection found that none of the commonly used rules or tools had good clinical utility (142).

Three of the cases reported in the UK as misdiagnosed swine flu, actually had *Plasmodium falciparum* malaria (137, 138). This may have been related to the original case definition, which did not require information on possible malaria exposure or travel to a malaria endemic country to be collected (136), although later versions of the case definition did exclude those who had recently been to a malarial area from telephone triage. In reviews of febrile illness in travellers returning from malarial endemic areas, malaria was the most common diagnosis (143-145), but a significant minority of patients in these reviews had influenza infection. There are reports of patients with influenza mimicking malaria (146) and at least one case report of a patient presenting with an influenza like illness who had a final diagnosis of malaria (147). As was shown in some of the case reviews of pandemic H1N1 infection, atypical features, notably diarrhoea and vomiting can be present (21, 30, 123, 125, 127) and may add to the diagnostic uncertainty in these cases. In this context, the data from paper 6 in this thesis (148), is of use in guiding assessment of patients, during periods of influenza activity, who are at risk of malaria. As may be expected, upper respiratory symptoms were more common in the influenza group, whilst all other symptoms were not able to distinguish clinically between influenza and malaria infection. It would therefore seem sensible that in the absence of respiratory symptoms, patients should be evaluated for other causes of their presumed influenza illness, if they have a relevant travel or exposure history.

6. Future areas of study

Research into influenza vaccines and the potential to generate heterosubtypic immunity is an ongoing area of active research. Co-administration of the novel vaccine MVA-NP+M1 together with seasonal influenza vaccine has recently been studied (149), as a strategy to boost the humoral response to TIV and shows promise in potentially achieving both humoral and cellular immune responses against influenza A viruses. This approach, of using both poxvirus and protein based vaccine to achieve greater cellular and humoral immune responses, has been previously demonstrated in a murine model of hepatitis B vaccination (150). This combination vaccination strategy may abrogate the reduced efficacy seen when there is a mismatch between the circulating virus and that included in the seasonal trivalent vaccine (41). Another development with MVA-NP+M1 is its use in combination with a novel adenovirus expressing the same antigens (103). This may be of use in vaccinees who have little in the way of pre-existing T-cell responses to influenza, who would require a prime boost strategy, with adenovirus priming followed by boosting with MVA-NP+M1. The extension of studies of MVA-NP+M1 into other at risk groups for influenza, such as those with chronic respiratory conditions is another area that would be of interest. Other vaccines and vaccine antigens are in development and the hope is that a potent, heterosubtypic vaccine can be developed that can be of use in both seasonal and pandemic influenza prevention. As there are further cases of zoonotic transmission of avian influenza viruses, such as H9N2 (151) and H5N1 (152), the need for a pre pandemic vaccine that could at least reduce the impact of a potentially severe pandemic is particularly pressing.

One remaining area of data that is currently being analysed and prepared for submission for publication, from the influenza challenge study described in paper 3 of this thesis (102), consists of transcriptomic profiling of both vaccinated and non-vaccinated volunteers. Early analysis of this data suggests that it may provide clues as to the reasons why some patients have more severe symptoms than others who shed similar levels of virus after challenge, and therefore help in quantifying symptoms in influenza challenge studies in a more objective manner. Other potential uses of transcriptomic profiling include differentiating between viral and bacterial illness (111), thereby potentially allowing better targeting of antibiotics and antivirals, and potentially in prognostication as the immune

inflammatory response to pandemic H1N1 has been shown to be a major determinant of outcome (153, 154).

Another aspect of the research described within this thesis is the search for correlates of cellular immunity to influenza infection. The most recent studies on this are conflicting on the relative roles of CD4 and CD8 T-cells (82, 83), as well as the overall role of T-cell responses after vaccination (54, 78) and future research should aim to clarify this area. The role of other aspects of the cellular immune response, such as natural killer cells is also under investigation (155). The reduced vaccine efficacy seen in the elderly is being studied, and the role of immunosenescence and in particular chronic infection with cytomegalovirus is a potentially fascinating area (156, 157).

With regard to the clinical assessment of influenza illness and severity assessment, data from the 2009 pandemic continues to be analysed together with the identification of factors associated with complicated disease and the generation of assessment algorithms (158). A recent paper from the USA found that outcomes were similar in patients who had confirmed influenza B virus infection, compared with influenza A infection (159), and this may help in simplifying assessment tools. How relevant this data is as we move further away from the pandemic and into the situation where the 2009 H1N1 virus becomes the main seasonal circulating influenza A strain is a point of conjecture. Further development of prognostic tools to allow the community based treatment of patients with influenza would be useful, such as that described in paper 5 (30) of this thesis.

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8. Published papers

8.1 Paper 1 - Potent CD8+ T-Cell Immunogenicity in Humans of a Novel Heterosubtypic Influenza A Vaccine, MVA-NP+M1

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Background. Influenza A viruses cause occasional pandemics and frequent epidemics. Licensed influenza vaccines that induce high antibody titres to the highly polymorphic viral surface antigen haemagluttinin must be reformulated and re-administered annually. A vaccine providing protective immunity to the highly conserved internal antigens could provide longer lasting protection against multiple influenza subtypes.

Methods. We prepared a Modified Vaccinia virus Ankarra (MVA) vector encoding nucleoprotein and matrix protein 1 (MVA-NP+M1) and conducted a phase I clinical trial in healthy adults.

Results. The vaccine was generally safe and well tolerated, with significantly fewer local side effects after intramuscular rather than intradermal administration. Systemic side effects increased at the higher dose in both frequency and severity, with 5 out of 8 volunteers experiencing severe nausea / vomiting, malaise or rigors. Ex-vivo T-cell responses to NP and M1 measured by IFN-γ ELISPOT were significantly increased after vaccination (pre vaccination median of spot forming units/million peripheral blood mononuclear cells, post vaccination peak response median 339, 443 and 1443 in low dose intradermal, low dose intramuscular and high dose intramuscular groups, respectively) and the majority of the antigen specific T-cells were CD8+.

Conclusions. We conclude that the vaccine was both safe and remarkably immunogenic, leading to frequencies of responding T-cells that appear to be much higher than those induced by any other influenza vaccination approach. Further studies will be required to find the optimum dose and to assess whether the increased T cell response to conserved influenza proteins results in protection from influenza disease.

Licensed influenza vaccines, whether inactivated or live attenuated, are designed to induce humoral immunity to haemagglutinin (HA). Seasonal influenza vaccines are a mixture of A/H1N1, A/H3N2, and B antigens. Vaccine effectiveness is 70%-90% when the circulating virus is well matched to the vaccine, but may fall below 50% when the circulating strain has drifted significantly from the vaccine strain (1), particularly in people over 60 years of age (2). Annual revaccination is required to maintain immunity against seasonal influenza viruses.

Fears of an H5N1 pandemic resulted in the generation and testing of H5-specific vaccines, which may require the use of an adjuvant or multiple doses to achieve a protective level of immunity following vaccination (3). H5N1 viruses have continued to mutate in avian populations, and in clinical trials of unadjuvanted H5 vaccine, serological cross-reactivity to variant H5 viruses even within the same clade was only 20%-30% (4), although use of an adjuvant may improve this. Since swine origin H1N1 began to circulate in humans in April 2009, vaccine manufacturers have produced pandemic-specific vaccines, and the first doses became available in October 2009, 6 months after the virus was first identified.

Clearly, a vaccine that could provide heterosubtypic protection against all influenza A viruses would be of great benefit, and if effective and widely used, could prevent another pandemic from occurring. The efficacy of influenza vaccines designed to induce subtype cross-reactive T cells to internal influenza antigens such as nucleoprotein (NP), which is highly conserved between all influenza A subtypes, has been demonstrated in many species of animal model (5-8) and this approach has the potential to replace or supplement seasonal and pandemic-specific vaccination in humans. Influenza challenge studies in humans with low neutralising antibody titres to the challenge virus (measured by HA inhibition assay) have demonstrated a negative correlation between T cell response to viral antigens and influenza disease and viral shedding (9). Protection is thought to be mediated chiefly by CD8+ T cells, but protective immunity is short lived (10), although re-exposure to influenza virus within a few years of the first infection may result in a subclinical infection and boosting of the T cell response. Lee et al (11) reported that memory T cells recognising influenza antigens were detected in over 90% of those tested,

and showed cross-recognition of at least one H5N1 internal protein. The magnitude of the responses varied considerably, and is presumably related to the time elapsed since the most recent exposure to influenza virus. However, low-level memory T cell responses to influenza antigens have the potential to be boosted to protective levels, by further exposure to the virus or by vaccination. Live attenuated influenza vaccines have been shown to induce modest T cell responses in children, but did not significantly boost T cell responses to influenza in adults with T cell responses induced by natural exposure (12).

Modified vaccinia virus Ankara (MVA) is a highly attenuated virus that has been shown to boost T cell responses to recombinant antigens encoded by the virus in many clinical studies aimed at developing new vaccines for malaria, human immunodeficiency virus (HIV) and tuberculosis (TB). MVA has an excellent safety profile, and has been tested in children (13), as well as HIV-positive (14) and latently TB-infected individuals (15). MVA has been used to boost both CD4+ and CD8+ responses primed by prior DNA, fowlpox (16), adenovirus (17), or Bacille Calmette-Guerin immunisation (18) or HIV infection (14). Since adults have been primed by prior exposure to influenza, MVA expressing conserved internal antigens of influenza such as NP and matrix protein 1 (M1) could be used to boost cross-reactive T cell responses, providing broad immunity to all subtypes of influenza A. An illustration of the conservation of the vaccine antigens is given in Table 1, showing the identity and divergence of the amino acid sequences of NP and M1 in the vaccine MVA-NP+M1 and human isolates of H3N2, H1N1, H5N1 and swine origin H1N1. The identity and divergence of HA are given for comparison. The high degree of identity with H3N2 is not surprising since the vaccine antigens are derived from the H3N2 virus A/Panama/2007/99, but both antigens are more than 90% identical with homologues from seasonal H1N1, swine origin H1N1 and H5N1 viruses, whereas identity drops as low as 43% between the HA proteins of the same 4 viruses. We now report on the safety and immunogenicity of MVA-NP+M1, a vaccine designed to boost pre-existing T cell responses to conserved influenza antigens, in a phase I clinical study in healthy adult volunteers.

Table 1. Sequence Identity (Top) and Divergence (Bottom) Between Antigens in MVA-NP1M1 and Other Influenza A Viruses

A : Nucleoprotein

	Vaccine	**H3N2**	**H1N1**	**H5N1**	**SO H1N1**
Vaccine	X	98.0	91.8	91.4	90.2
H3N2	2.0	X	91.4	90.8	89.8
H1N1	8.7	9.2	X	92.0	90.0
H5N1	9.2	9.9	8.5	X	93.6
SO H1N1	10.5	11.0	10.8	6.7	X

B : Matrix Protein 1

	Vaccine	**H3N2**	**H1N1**	**H5N1**	**SO H1N1**
Vaccine	X	99.2	94.9	92.9	92.1
H3N2	0.8	X	95.7	92.9	92.1
H1N1	5.3	4.5	X	93.3	93.7
H5N1	7.5	7.5	7.1	X	96.0
SO H1N1	8.4	8.4	6.6	4.1	X

C : Haemagglutinin

	Vaccine	**H3N2**	**H1N1**	**H5N1**	**SO H1N1**
Vaccine	X	N/A	N/A	N/A	N/A
H3N2	N/A	X	42.6	44.0	42.8
H1N1	N/A	100.0	X	79.3	63.1
H5N1	N/A	97.3	24.2	X	63.8
SO H1N1	N/A	100.0	50.4	49.1	X

NOTE. Calculated using DNAStar MegAlign 8.0 after Jotun Hein alignment. Percent identity 5 (Matches x 100)/Length of aligned region (with gaps); divergence is calculated by comparing sequence pairs in relation to the reconstructed phylogeny. Viruses are H3N2: A/Pennsylvania/PIT08/2008 (NP: CY035057, M1: CY035055, HA: CY035054), H1N1: A/Washington/AF06/2007 (NP: CY037330, M1: CY037328, HA: CY037327), H5N1: A/Beijing/01/2003 (NP: EF587278, M1: EF587280, HA: EF587277), SO H1N1: A/Canada-NS/ RV1535/2009 (NP: FJ998216, M1: FJ998210, HA: FJ998207).

MATERIALS AND METHODS

Sequence Alignments. Sequences were obtained from the National Centre for Biotechnology Information GenBank and aligned using Lasergene DNAStar 8.0 MegAlign, Jotun Hein method.

Vaccine Design and Manufacture. The vaccine antigen expressed from MVA consists of the complete NP and M1 from A/Panama/2007/99 joined by a 7 amino acid linker sequence, and is expressed from the Vaccinia P7.5 promoter inserted at the thymidine kinase locus of MVA. Generation of the recombinant virus and subsequent Good Manufacturing Practice (GMP) manufacture used primary chick embryo fibroblast (CEF) cells. GMP manufacture and release testing of the vaccine were carried out by Impfstoffwerk (Dessau-Tornau, Germany).

Study Population. Twenty-eight subjects were recruited for immunisation studies under a protocol approved by the United Kingdom's Medicines and Healthcare products Regulatory Agency and Gene Therapy Advisory Committee and were enrolled only after obtaining written informed consent (www.clinicaltrials.gov, identifier: NCT00942071). Inclusion criteria required volunteers to be aged 18-50 years, resident in the Oxford area, and seronegative for HIV antibodies, hepatitis B surface antigen and hepatitis C antibodies. Women who were pregnant or lactating, and volunteers who had previously received an MVA (but not vaccinia) vaccine, or who had a history of egg allergy or anaphylaxis following vaccination, were excluded. No information about prior seasonal influenza vaccination was recorded, but volunteers did not fall into the target population for vaccination within the UK and were unlikely to have received vaccination.

Vaccination and Follow-up Regime. Following receipt of information about the study, volunteers attended a screening visit to assess their suitability for the study. Each group was completed and vaccine safety assessed before enrolling the next group. All eligible volunteers were enrolled into the next available group. A single vaccination was administered at a subsequent visit, with the dose and route of vaccination varying

between the study groups. Group 1 received 5 x 10^7 pfu intradermally (dose volume 385 microlitres), group 2 received the same dose intramuscularly, and group 3 received 2.5 x 10^8 pfu intramuscularly (dose volume 1920 microlitres). Blood was drawn to assess the T cell response to NP and M1 on the day of vaccination and 1,3,8,12,24 and 52 weeks after vaccination. Volunteers also attended a follow-up visit 2 days after vaccination; adverse events were elicited by open questions at that visit and all visits up to week 12 and were also recorded on a diary card by the volunteer for the first week after vaccination. Mild events were defined as awareness of a symptom that was easily tolerated, moderate as discomfort enough to cause interference with usual activity, and severe as incapacitating, inability to perform usual activities, requiring absenteeism or bed rest. Information about influenza-like illness was also recorded, with no volunteer reporting symptoms within the first 3 weeks following vaccination, and very few reports of coryzal illness at later time points. Vaccinations were carried out from August to November 2008 (group 1), February to March 2009 (group 2) and March to May 2009 (group 3). Circulating seasonal influenza A strains during this period were H3N2 – A/Brisbane/10/2007 and H1N1 – A/Brisbane/59/2007.

Ex Vivo IFN-γ ELISPOT. Ex vivo interferon-gamma enzyme-linked immunosorbent spot (IFN-γ ELISPOT) assays were performed using cryopreserved peripheral blood mononuclear cells (PBMCs). PBMCs were cryopreserved in fetal calf serum (FCS) (Biosera Ltd) with 10% dimethyl sulfoxide (Sigma) at -80°C in a Mr Frosty container, then transferred and stored in liquid nitrogen. PBMCs were thawed quickly in warm R10 (R10: RPMI 1640 with 10% FCS, 100IU/ml penicillin, 0.1mg/ml streptomycin (all Sigma) and 2mM L-glutamine (GIBCO / Invitrogen), washed and re-suspended in R10 with 2µl/ml of 25U/ml Benzonase nuclease (Novagen) and left to rest overnight at 37°C. The following day the cells were washed and counted for use in the assays. The ex vivo IFN-γ ELISPOT was carried out as previously described (19). Fifteen- to 20-mer peptides overlapping by 10 amino acid residues, spanning the whole of the NP+M1 insert, were used to stimulate PBMCs at a concentration of 10µg/ml. The peptides were split into 8 pools of 10 peptides; pools 1-6 contained peptides from the NP sequence, and pools 6-8 contained peptides from the M1 sequence. Fifty microliters of PBMC's (2 x 105 cells) and 50 µl of the peptides were added in triplicate. R10 was used as a negative control, and phytohaemagluttinin (PHA) at a final concentration of 10µg/ml was used as a positive control. Following an 18 to 20 hour incubation at 37°C, the ELISPOT plates were dried and read with an AID ELISPOT reader

(AID Diagnostika). The results are expressed as spot-forming units (SFUs) per million PBMCs, calculated by subtracting the mean R10 negative control response from the mean peptide pool response. To determine the ELISPOT response to the vaccine insert, the response to each peptide pool was summed following background subtraction. Plates were excluded if a response of over 100 SFU/ million PBMCs was seen in the R10 wells or under 1000 SFU in the PHA wells.

Intracellular Cytokine Staining. One to 2 million cryopreserved PBMCs were stimulated with a single pool of all NP+M1 peptides at a final concentration of 4 µg/ml and 1 µg/ml of co-stimulatory antibodies αCD28 and αCD49d (BD Pharmingen). Cells were incubated for 18 hours at 37°C. After the first 2 hours of incubation, 10 µg/ml brefeldin A and monensin (eBiosciences) was added. PBMCs were stained with: CD3 Alexa Fluor 700 (eBioscience-UCHT1), CD8-APC-AF780 (eBioscience-RPAT8), CD4-QD655 (Invitrogen-S3.5), IFN-γ FITC (eBioscience-4S.B.3), CD14 Pacific Blue (Invitrogen-TuK4), CD19 Pacific Blue (Invitrogen-SJ25-C1), and VIVIV Pacific Blue (Invitrogen). Over 300,000 gated lymphocyte events were acquired on a Becton Dickinson LSRII flow cytometer using FACSDiva software (BD Biosciences) and analysed using Flow Jo, Version 8.3 (Tree Star Inc). Unstained cells and single stained anti-human compensation beads (BD Biosciences) were used as controls to automatically calculate compensation. All antibodies were titrated for optimal staining.

Statistical Analysis. Fishers exact test was used to detect significant differences in adverse events between the 3 vaccine groups. If such a difference existed, groups 1 and 2 were compared and the difference in proportions presented, and similarly for groups 2and 3. Non-parametric tests were used to determine differences in the ELISPOT data; Wilcoxon signed rank test was performed to test for differences in the ELISPOT responses between time points within a vaccine group, and Mann-Whitney U test was performed to detect differences in ELISPOT responses between different vaccine groups.

MVA-NP+M1 is Safe in Healthy Volunteers. Volunteers were given a single dose 5 x 10^7 pfu intradermally (group 1, 12 subjects), 5 x 10^7 pfu intramuscularly (group 2, 8 subjects) or 2.5 x 10^8 pfu intramuscularly (group 3, 8 subjects). Adverse events are presented in Figure 1. Volunteers receiving the vaccine via the intramuscular route, at either dose, experienced significantly less erythema, itch, swelling and warmth at the injection site than those vaccinated intradermally, regardless of the vaccine dose. All local adverse events were grade 1 severity apart from 1 volunteer in group 1 and 2 volunteers in group 2, who each experienced one grade 2 adverse event. No significant differences in systemic adverse events were reported by the volunteers receiving the low-dose vaccine by either route (no grade 3 adverse events in either group), but there was a significant increase in malaise, nausea/vomiting and rigors in the group receiving the high-dose vaccination with 5 volunteers experiencing 1 or more severe adverse events (Figure 1B).

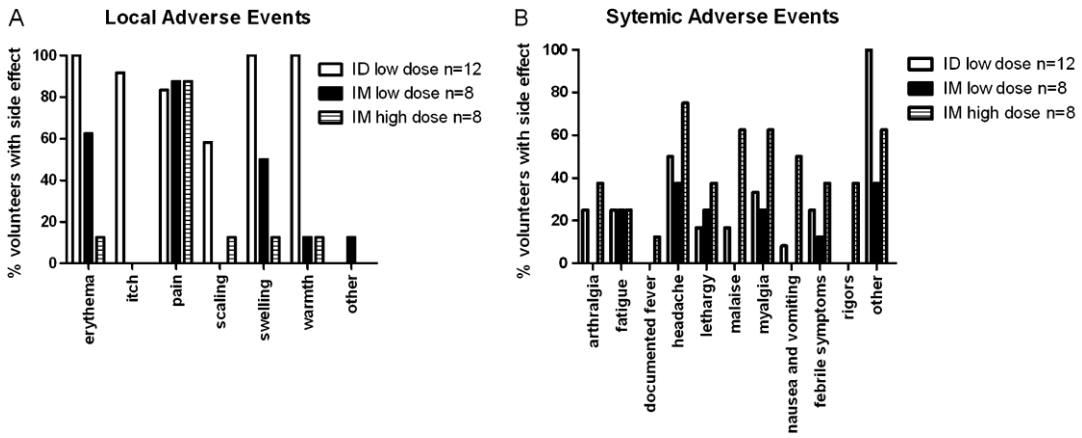

Figure 1. Local and systemic adverse events recorded after vaccination. Black: group 1 ($n = 12$). White: group 2 ($n = 8$). Striped: group 3 ($n = 8$). (A) Local adverse events. Significantly less ($P < .05$, Fisher's exact test) erythema, itch, swelling, and warmth at the injection site were detected in those receiving intramuscular vaccine than those vaccinated intradermally, regardless of the vaccine dose. Significantly less scaling was recorded in the low-dose compared with the high-dose intramuscular group. (B) Systemic adverse events. No significant differences in systemic adverse events were reported by the volunteers receiving the low-dose vaccine by either route, but there was a significant increase in malaise, nausea/vomiting, and rigors in the group receiving the high dose ($P < .05$, Fisher's exact test). Severe adverse events only occurred in the high-dose group, with 2 volunteers reporting severe pain at the injection site, 1 reporting malaise, 1 vomiting, 2 rigors, and 1 sweating. All severe adverse events resolved within 48 hours of vaccination, apart from 1 volunteer reporting severe pain at the injection site on the 3 days following vaccination. The majority of mild and moderate adverse events also took place within 48 hours of vaccination, although mild erythema at the injection site lasted for up to 49 days for those receiving intradermal vaccination.

MVA-NP+M1 Vaccination Boosts IFN-γ Secreting Antigen-Specific Tcells.

Ex vivo IFN-γ ELISPOT responses to the whole NP and M1 vaccine insert were measured in cryopreserved PBMCs at baseline (week 0) and at 1, 3, 8, 12, 26, and 52 weeks after immunization (Figure 2A-C). All volunteers had measurable responses to NP and M1 prior to vaccination (median 123 SFU / million PBMC). A significant increase in the number of SFUs detected following vaccination was seen in all 3 groups as measured by Wilcoxon signed rank test at weeks 1 and 3. Responses were also significantly above pre-vaccination level at weeks 8 and 12 for group 1, and weeks 8, 12 and 24 for group 3. The route of immunization did not appear to affect the magnitude of the immune response at low dose (no significant difference between groups 1 and 2) whereas the increase in dose from 5 x 10^7 pfu (group 2) to 2.5 x 10^8 pfu (group 3) resulted in significant increase in immune response at weeks 1, 3, 8, 12 and 24.

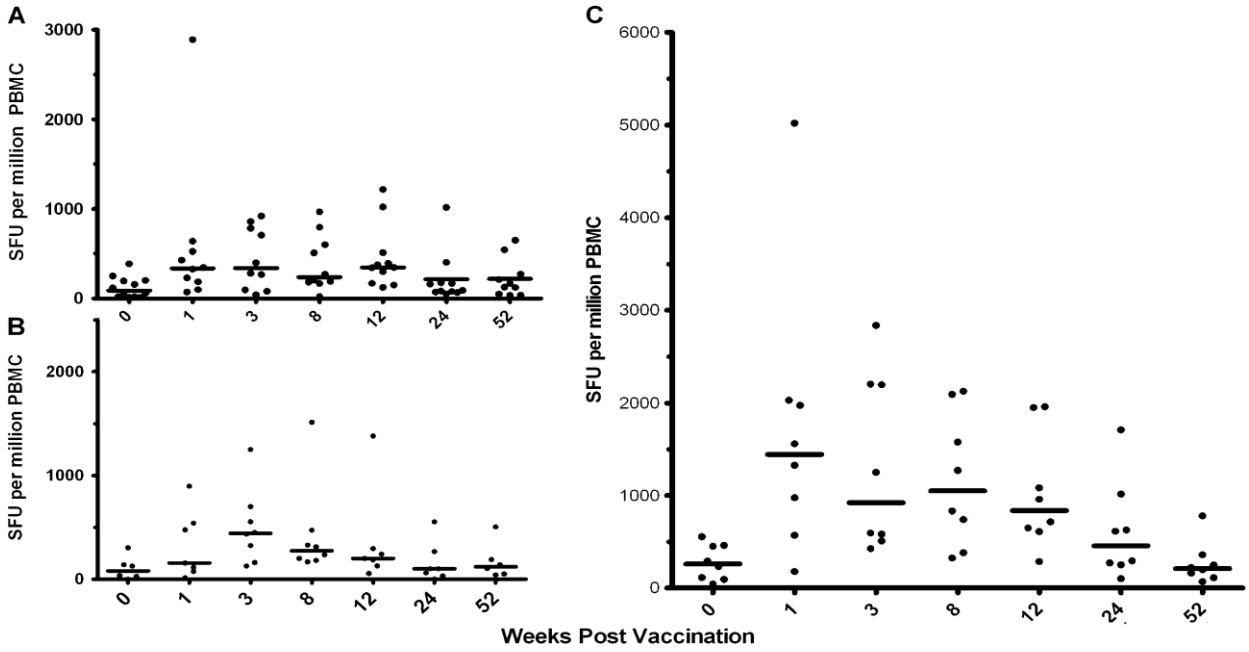

Figure 2. Ex vivo IFN-*g* ELISPOT responses to the vaccine insert. Median with individual ex vivo IFN-γ ELISPOT responses from vaccinated volunteers at baseline (week 0), and weeks 1, 3, 8, 12, 24, and 52 weeks after immunization. (A) group 1; (B) group 2; (C) group 3. Wilcoxon signed rank test was used to determine significant differences in the post- and prevaccination time points. (A) week 1, *P* = .0059; week 3, *P* = .0098; week 8, *P* =.0078; week 12, *P* = .0049. (B) week 1, *P* = .0313; week 3, *P* = .0313. (C) week 1, *P* = .0078; week 3, *P* = .0078; week 8, *P* = .0078; week 12, *P* = .0078;week 24, *P* = .023. Significant differences were detected between groups 2 (B) and 3 (C) at all postvaccination time points apart from week 52 (Mann- Whitney *U* test: week 1, *P* = .006; week 3, *P* = .04; week 8, *P* = .01; week 12, *P* = .02; week 24, *P* = .012).

Vaccination Boosts Both CD4+ and CD8+ Antigen-Specific T cells. Intracellular cytokine staining (ICS) analysis was carried out to determine whether the IFN-γ detected in the ex vivo ELISPOT was produced by CD3+ CD4+ or CD3+ CD8+ T cells. ICS was carried out at week 0, week 1 and week 8 on cryopreserved PBMCs from all volunteers in group 3. The CD4+ and CD8+ T cell responses following background subtraction are shown in figure 3. A significant increase in IFN-γ production from CD8+ T cells was detected following vaccination at week 1 and week 8. The percentage of antigen-specific CD8+ cells producing IFN-γ was higher than the corresponding of CD4+ T cells both before and after vaccination. Interlukin-2 and tumor necrosis factor alpha (IL-2 and TNF-α) production and CD107a expression were also analyzed in the CD4+ and CD8+ populations, revealing quadruple, triple, double and single functional cells in both populations (Figure 4). CD107a, a marker of degranulation and cytotoxicity, was present both with and without IFN-γ.

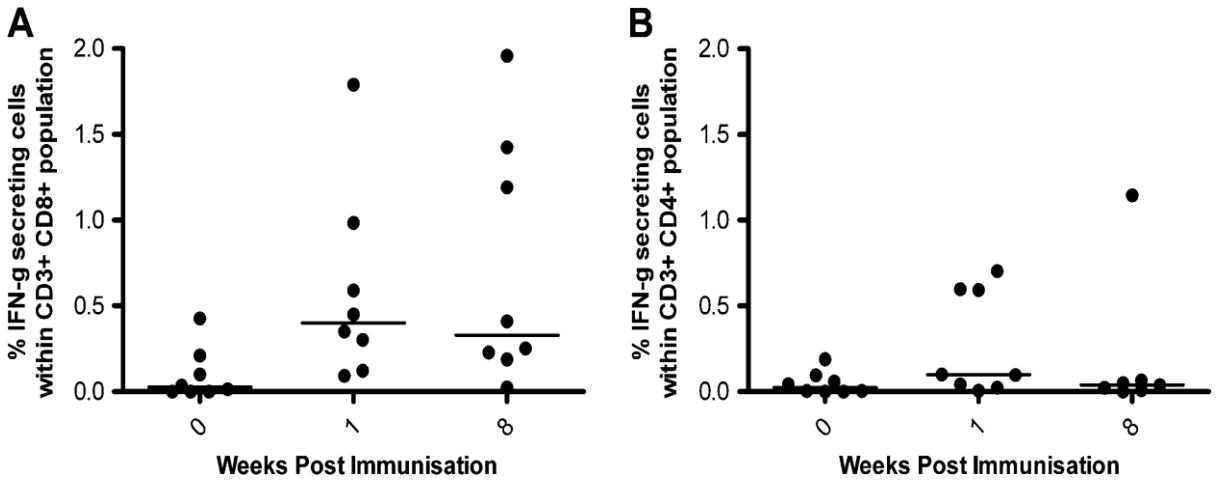

Figure 3. $CD3^+CD4^+$ and $CD3^+CD8^+$ IFN-γ responses to vaccine insert as measured by intracellular cytokine staining. Intracellular IFN-γ responses after background subtraction in (A) $CD3^+CD8^+$ and (B) $CD3^+CD4^+$ cell populations stimulated with 1 pool of peptides spanning the complete NP+M1 vaccine insert. Volunteers in group 3 were tested at weeks 0, 1, and 8. Median % IFN-γ within $CD3^+CD8^+$ cells at week 1 = .4% and week 8 =.33%; median % IFN-γ cells within $CD3^+CD4^+$ population at week 1 = .098% and week 8 = .039%

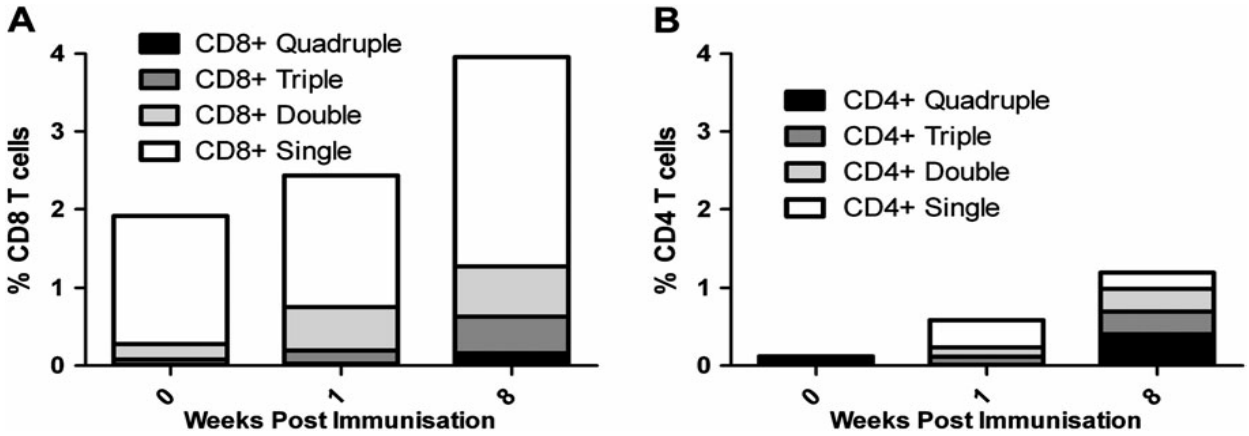

Figure 4. IFN-γ, IL-2, TNF-α, and CD107a multifunctional cells detected by ICS in $CD3^+CD8^+$ and $CD3^+CD4^+$ populations. Mean percentage of quadruple (black), triple (dark gray), double (light gray), and single (white) functional cells detected within the $CD8^+$ (A) and $CD4^+$(B) populations. Within the $CD8^+$ population, the most frequently detected triple positive cells were $CD107a^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$; the most frequently detected double positive cells were $CD107a^+TNF\text{-}\alpha^+$; and $CD107a^+$ cells were the most frequently detected single positive cells. Within the $CD4^+$ cells, the most frequently detected triple positive cells were $CD107a^+IFN\text{-}\gamma^+IL\text{-}2^+$; the most frequently detected double positive cells were $IFN\text{-}\gamma^+IL\text{-}2^+$; and $TNF\text{-}\alpha^+$ cells were the most frequently detected single positive cells. At all time points the frequency of antigen-specific cytokine positive cells was greater in the $CD8^+$ population (week 0, $CD8^+$ = 1.92% and $CD4^+$ = .12%; week 1, $CD8^+$ = 2.43% and $CD4^+$ = .58%; week 8, $CD8^+$ = 3.96% and $CD4^+$ = 1.19%).

DISCUSSION

We report here the first clinical study of a novel influenza vaccine designed to boost cross-reactive immune responses to all influenza A subtypes. Many studies have reported intradermal vaccination with MVA, and the side effect profile seen with MVA-NP+M1 is comparable (20). The same dose administered by the intramuscular route resulted in significantly fewer local, but not systemic, adverse events. At the higher dose of 2.5×10^8 pfu administered as an intramuscular injection, there was an increase in both the frequency and severity of systemic adverse events compared with the lower dose of 5×10^7 pfu. For further studies with this vaccine the dose will be reduced to 1.5×10^8 pfu. The magnitude of the immune response to vaccination determined by ex vivo IFN-γ ELISPOT did not differ with the route of administration, but increased at the higher dose.

ICS analysis for IFN-γ production by $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells was also carried out in the high-dose intramuscular group and showed that more antigen-specific $CD3^+$ $CD8^+$ T cells than $CD3^+$ $CD4^+$ T cells were present after vaccination. IL-2, TNF-α and CD107a were also produced by antigen-specific cells. Further studies are required to determine which T cell phenotypes, whether lytic or cytokine producing, are capable of prevention of disease following exposure to influenza virus.

A vaccine that boosts cross-reactive T cell responses to conserved internal antigens of influenza has the potential to modify or prevent disease and virus shedding in vaccinees, thus reducing morbidity and transmission whether the virus is one that continually circulates in humans or is a different subtype with the potential to cause a pandemic. Vaccines based on HA protein must be produced not only for each virus subtype, but for the continually evolving sequences within each subtype. MVA-vectored vaccines can be produced at a large scale for human vaccination and are safe for use. MVA-NP+M1 could be used alone, or in combination with an anti-HA antibody producing component, to provide broad protection against all influenza A viruses, thereby improving vaccine efficacy over that currently achieved, particularly in seasons when the circulating virus has drifted from the vaccine strain, and to provide protection when a global pandemic occurs, regardless of the virus subtype.

Currently the magnitude of the T cell response to NP and M1 required to prevent influenza disease in humans is not known. However, the magnitude of the induced T cell responses measured here are noteworthy. A median response of 1443 SFU / million PBMCs at the peak time point substantially exceeds the T cell responses induced in any of the large numbers of phase I and II trials of potent vectored vaccines in HIV, malaria and cancer (21,22). In the STEP trial of an adenovirus vectored vaccine against HIV-1, the geometric mean T cell response at peak was around 300 SFU / million PBMCs to the vaccine antigens. The much higher immunogenicity identified here likely results from the level of T cell response prior to vaccination attributable to natural influenza virus exposure, combined with the remarkable boosting ability of MVA-vectored vaccines. A similar potent boosting of pre-existing T cell responses and induction of poly-functional T cell responses is observed with an MVA vector encoding a TB antigen (23). In contrast, a trial of the cold-adapted influenza virus vaccine, FluMist, found that although it could induce modest T cell responses in children, it did not significantly boost T cell responses in adults with T cell responses induced by natural exposure (12).

A further notable finding is the greater $CD8^+$ than $CD4^+$ T cell response after vaccination. In the only other previous example of vaccine-induced T cell response exceeding 1000 SFU / million PBMCs to an antigenic insert, the response was predominately of $CD4^+$ T cells (24). This reflects the proportions of $CD4^+$ and $CD8^+$ T cells detected prior to vaccination in each case and adds growing evidence that MVA vectors can boost both $CD4^+$ and $CD8^+$ T cells effectively in humans.

Further planned studies will address the ability of this MVA vaccine to boost pre-existing T cell responses to the conserved influenza antigens NP and M1 in an extended age range, as well as the efficacy of the vaccine in preventing influenza disease and virus shedding via influenza virus challenge studies.

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8.2 Paper 2 - A T Cell Inducing Influenza Vaccine for the Elderly: Safety and Immunogenicity of MVA-NP+M1 in Adults Aged over 50 Years

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ABSTRACT

Background. Current influenza vaccines have reduced immunogenicity and are of uncertain efficacy in older adults. We assessed the safety and immunogenicity of MVA-NP+M1, a viral vectored influenza vaccine designed to boost memory T-cell responses, in a group of older adults.

Methods. Thirty volunteers (aged 50–85) received a single intramuscular injection of MVA-NP+M1 at a dose of 1.5×10^8 plaque forming units (pfu). Safety and immunogenicity were assessed over a period of one year. The frequency of T cells specific for nucleoprotein (NP) and matrix protein 1 (M1) was determined by interferon-gamma (IFN-γ) ELISPOT, and their phenotypic and functional properties were characterized by polychromatic flow cytometry. In a subset of M1-specific CD8+ T cells, T cell receptor (TCR) gene expression was evaluated using an unbiased molecular approach.

Results. Vaccination with MVA-NP+M1 was well tolerated. ELISPOT responses were boosted significantly above baseline following vaccination. Increases were detected in both CD4+ and CD8+ T cell subsets. Clonality studies indicated that MVA- NP+M1 expanded pre-existing memory CD8+ T cells, which displayed a predominant CD27+CD45RO+CD57-CCR7- phenotype both before and after vaccination.

Conclusions. MVA-NP+M1 is safe and immunogenic in older adults. Unlike seasonal influenza vaccination, the immune responses generated by MVA-NP+M1 are similar between younger and older individuals. A T cell inducing vaccine such as MVA-NP+M1 may therefore provide a way to circumvent the immunosenescence that impairs routine influenza vaccination.

INTRODUCTION

Winter epidemics of influenza in the UK have caused 7,000– 25,000 deaths in the past decade (1999–2010) (1). In addition, influenza infection exerts pressure on healthcare systems and results in substantial economic losses. The burden of disease in developed countries disproportionately affects the elderly, with approximately 90% of influenza-associated excess deaths occurring among people aged 65 years and older (2). Indeed, in those over the age of 75 years, 25–81% of all deaths in the UK in the last decade have been attributed to influenza virus infection (1). Government-funded vaccination programmes for influenza exist in many countries and include elderly individuals in their target populations (3]) Unfortunately, the rates of seroprotection and seroconversion following vaccination are significantly lower in the elderly (4). A recent systematic review found vaccine efficacy was 59% in adults aged under 65 years, but no trials assessing protection from laboratory-confirmed influenza have been conducted in subjects aged over 65 years (5).

In the elderly, immunosenescence can negatively impact the ability of the immune system to mount an effective immune response to new pathogens and vaccines. Characteristics of immunosenescence include: (i) a decrease in B cell function, which is thought to result from defective T cell help; (ii) thymic involution and an associated reduction in naive T cell output; (iii) expansion of selected memory T cell clones driven by persistent viral infections such as CMV (reported to affect up to 90% of elderly individuals) (6) and; (iv) increases in anergic CD28 T cells and regulatory T cells (7). Accordingly, there is an urgent need for an effective influenza vaccine targeted to the requirements of the ageing immune system.

In addition to seasonal epidemics, influenza can cause pandemics, typically following a viral antigenic shift. Therefore, new vaccine candidates should ideally induce an element

of cross-strain (heterosubtypic) immunity (8). One approach is to generate high frequencies of CD8+ T cells directed against conserved influenza antigens (9). Viral-vectored vaccines elicit potent T cell responses and therefore represent a promising strategy in this regard. Modified vaccinia virus Ankara (MVA) is a highly attenuated strain of vaccinia virus in which viral replication is blocked at a late stage of virion assembly (10). Recombinant MVAs are therefore efficient single-round expression vectors, and have been used to prime or boost T cell responses to a diverse range of pathogen-specific and tumour-derived antigens. Previously, we have shown that a recombinant MVA expressing the nucleoprotein (NP) and matrix protein 1 (M1) sequences from a H3N2 strain of influenza A (termed MVA-NP+M1), was safe and immunogenic in young adults, significantly boosted T cell responses to NP and M1 [11] and has a protective effect against influenza challenge (12). Such an approach may help to circumvent the limitations of immunosenescence, by boosting pre-existing memory T cell responses rather than by attempting *de novo* priming from the naïve lymphocyte pool. We have now extended the Phase I trial into older adults, and demonstrate here that MVA-NP+M1 is safe and highly immunogenic in this population.

METHODS

Study Design. This was a Phase I open-label, non-randomized vaccine trial. The study was conducted at the Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford, Oxford, UK. The clinical trial protocol and supporting CONSORT checklist are available as Supplementary Information; see Protocol S1 and Checklist S1. The trial protocol was approved within the UK by the Medicines and Healthcare products Regulatory Agency and the Gene Therapy Advisory Committee. The stated objectives of the trial were to assess the safety and the cellular immune response of a new influenza vaccine, MVA-NP+M1, when administered to healthy volunteers. The trial was registered at www.clinicaltrials. gov (identifier: NCT00942071).

Participants. Thirty subjects were enrolled in three stratified age groups: 50– 59 years, 60–69 years and 70+ years (10 volunteers per group). Younger volunteers from our previous two clinical trials were used for comparative purposes (11,12). All volunteers were healthy adults, resident in the Oxford area, with negative pre-vaccination tests for

HIV antibodies, hepatitis B surface antigen and hepatitis C antibodies (see Supplementary Information: Protocol S1 for the full list of inclusion and exclusion criteria). Written informed consent was obtained in all cases. The planned sample size was 10 in each age group. This sample size should allow determination of the magnitude of the outcome measures, especially of serious and severe adverse events, rather than aiming to obtain statistical significance.

MVA-NP+M1 Vaccine. The vaccine was described previously and consists of MVA expressing the NP and M1 antigens from influenza A as a single fusion protein (11).

Procedures. Volunteers were vaccinated on the day of enrolment with a single intramuscular injection of MVA-NP+M1 at a dose of 1.5×10^8 pfu into the deltoid region of the arm. Blood was taken prior to the vaccination (week 0), and volunteers were observed for a period of 1 hour following the vaccination. Volunteers were given a digital thermometer, tape measure and symptom diary card to record their daily temperature, injection site reactions and solicited adverse events for 7 days. Two days after vaccination, volunteers were reviewed in clinic and assessed for potential adverse events. Volunteers were reassessed and blood samples were taken at subsequent visits, which occurred at 1, 3, 8, 12, 24, and 52 weeks post-vaccination.

Interferon-gamma ELISPOT. *Ex vivo* interferon-gamma enzyme-linked immunosorbent spot (IFN-γ ELISPOT) assays were performed using fresh peripheral blood mononuclear cells (PBMC) as described previously (13). Cells were washed and resuspended in RPMI 1640 containing 10% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin (all Sigma), and 2 mM L-glutamine (Life Technologies) (R10 medium). Peptides of 15–20 amino acids in length, overlapping by 10 amino acids and spanning the whole of the NP+M1 insert, were used to stimulate PBMC at a final concentration of 10 mg/ml in 8 pools of 10 peptides. R10 medium alone was used as a negative control, and a mixture of phytohaemagglutinin (PHA; 10 mg/ml) and staphylococcal enterotoxin B (SEB; 1 mg/ml) was used as a positive control. Each condition was assayed in triplicate using 2 x 10^5 PBMC in a final volume of 100µl per well. ELISPOT plates were incubated for 18-20

hours at 37°C. Developed and dried ELISPOT plates were analysed with an AID ELISPOT reader (AID Diagnostika). Results are expressed as spot-forming units (SFU) per million PBMC, calculated by subtracting the mean R10 negative control response from the mean peptide pool response and summing the net response for the 8 peptide pools. Plates were excluded if responses were greater than 100 SFU / million PBMC in the R10 wells, or less than 1,000 SFU / million PBMC in the PHA/SEB wells.

Intracellular Cytokine Staining. Intracellular cytokine staining (ICS) was performed using two different T cell staining panels at week 0, week 1 and week 3. The first panel detected Th1-type cytokine (IFN-γ, IL-2 and TNF-α) production and CD107a mobilization. The second panel detected CD107a mobilization, granzyme B expression, and the production of IL-10 and IL-17. Reagent details are provided in Table S1.

Fresh PBMC ($1–2 \times 10^6$) were stimulated for 18 hours at 37°C with either a single pool comprising of all the NP+M1 peptides at a final concentration of 4 mg/ml, SEB (1 mg/ml) or medium alone. The costimulatory monoclonal antibodies (mAbs) αCD28 and αCD49d (1 mg/ml each; BD Pharmingen) were added to panel 1, and αCD107a-PE-Cy5 (10 ml; eBioscience) was added to both panels. After 2 hours, brefeldin A and monensin (both eBioscience) were added. The cells were then washed, stained with the reagents listed in Table S1 according to standard procedures and acquired using an LSR II flow cytometer (BD Biosciences). Data were analysed using FlowJo version 9.4 (Tree Star, Inc.). Unstained cells and compensation beads (BD Biosciences) stained singly with the individual mAbs in each panel were used as controls to calculate compensation. All mAbs were titrated for optimal staining. Between 13,000 and 700,000 live, $CD14^-CD19^-$ lymphocyte events were collected and analysed per condition.

TCR Clonotyping. Cryopreserved PBMC were thawed and labelled with PE- conjugated GILGFVFTL/HLA A*0201 tetramer as described previously (14), then washed and surface stained with the directly conjugated mAbs listed in Table S1. Dead cells were excluded using LIVE/DEAD© Fixable Violet (Life Technologies), together with CD14+ and CD19+ events, in a single ‘‘dump’’ channel. Viable CD3+CD8+tetramer+ cells (918–5,000 per population) were sorted at 98% purity using a customized FACSAria II flow cytometer (BD Biosciences) and clonotypic analysis was conducted using a template-switch anchored RT-

PCR as described previously (14,15). The IMGT nomenclature was used to assign *TRB* gene usage (16).

Statistical Analysis. Statistical analysis was carried out using GraphPad Prism software version 5.04. The non-parametric Mann-Whitney U-test was employed to test for significant differences between groups of volunteers, and the non-parametric Wilcoxon signed rank test was used to test for significant differences between time points within the same group of volunteers.

RESULTS

Thirty volunteers were enrolled between April 06, 2010 and November 30, 2011 (Figure 1 and Table 1).

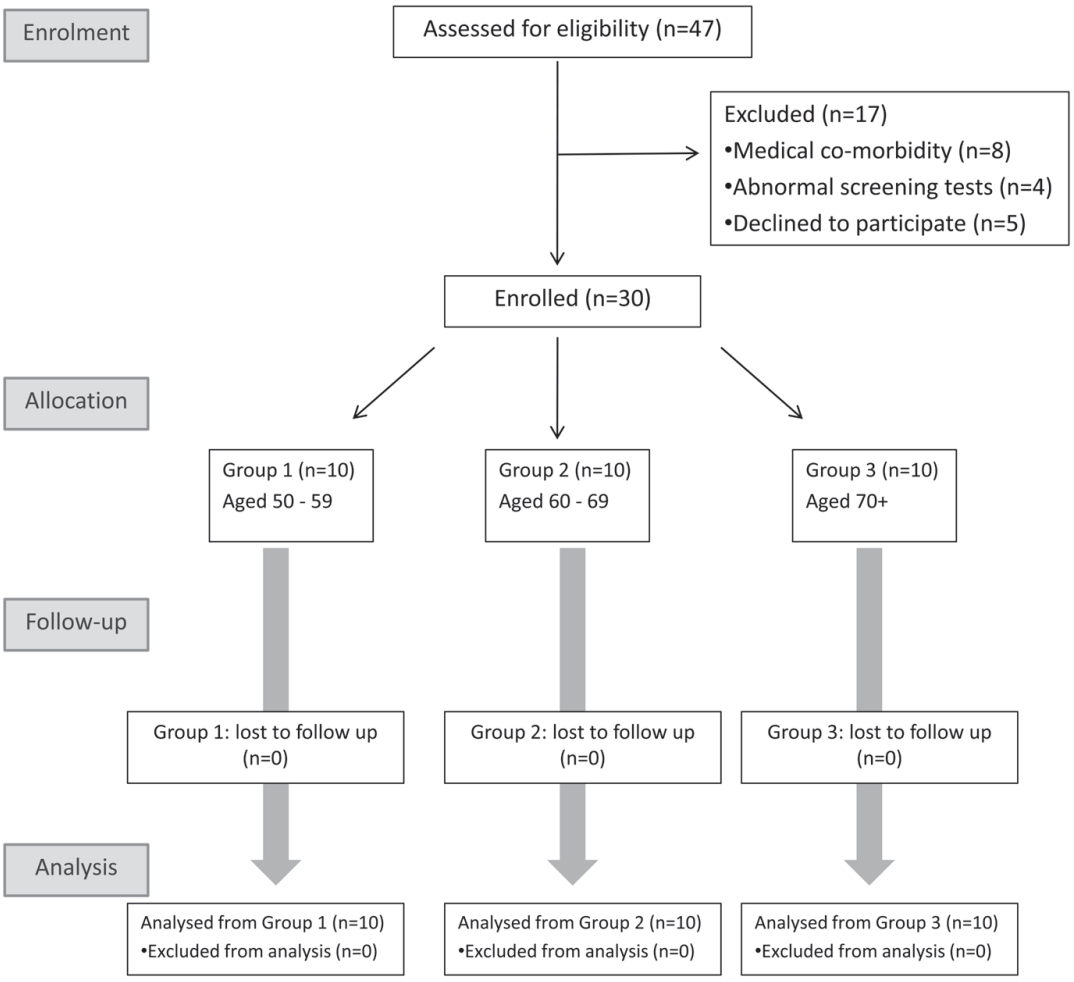


Figure 1. CONSORT flow diagram of the trial.

Table 1. Demographic Chacteristics of Volunteers Vaccinated in Each Cohort

Group	Age Range (years)	Mean Age (years)	Females
50-59	50-59	55.2	50%
60-69	60-66	63.3	70%
70+	72-85	79.0	50%

Vaccination was well tolerated and no serious vaccine-related clinical or laboratory adverse events were observed. The frequency of local and systemic adverse reactions is shown in Figure 2. All vaccine-related adverse events were either mild or moderate in severity.

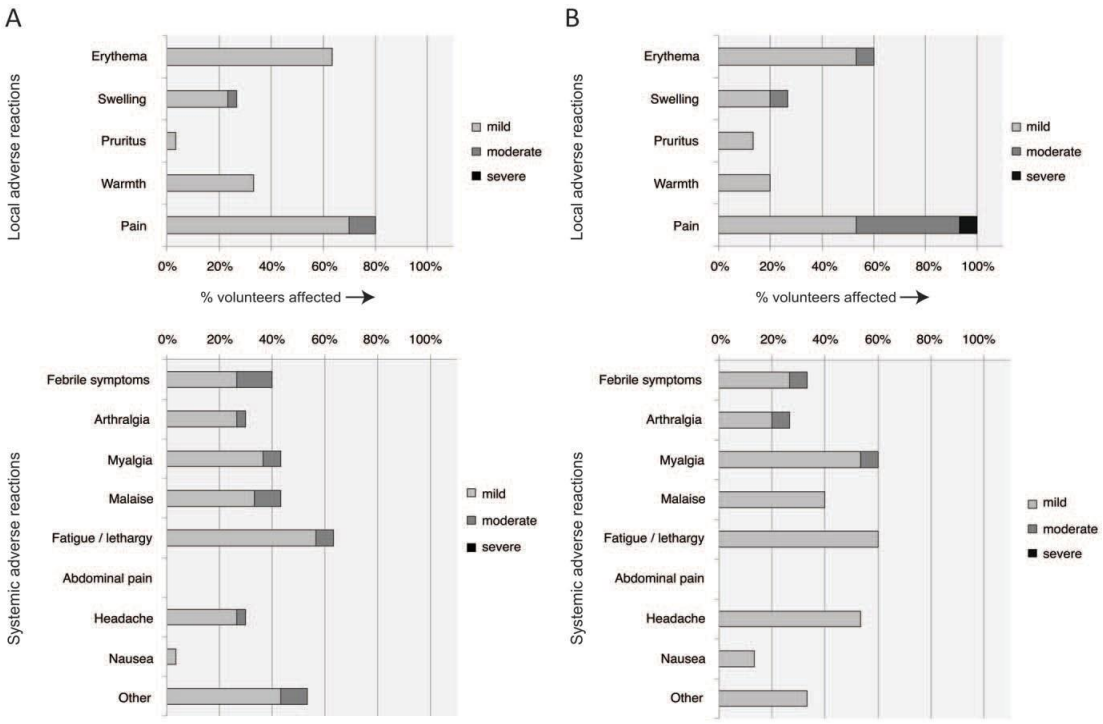


Figure 2. Frequency of local and systemic adverse events that were possibly, probably or definitely related to vaccination. (A) Volunteers aged 50+ (n=30). (B) Volunteers aged 18–45 (n=15). For both age groups pain was the most frequently recorded local adverse event followed by erythema. A similar pattern of systemic adverse events was observed in both age groups with the majority of solicited adverse events occurring in 20–60% of individuals. For volunteers aged 18–45, 85% of adverse events were mild; for volunteers aged 50+, 87% of adverse events were mild.

Figure 3a shows the T cell responses to the NP+M1 vaccine insert as measured by IFN-γ ELISPOT. All assays were conducted on fresh PBMCs as soon as they became available. As expected, T cell responses to NP and M1 were detected prior to vaccination with a median response of 188 SFU/million PBMC. These responses increased to a median of 1,603 SFU/million PBMC one week after vaccination, representing an 8.5-fold increase. When the data were stratified for age (group 1 = 50–59 years, group 2 = 60–69 years, group 3 = 70+ years), differences between the groups became apparent. In particular, T cell responses to NP and M1 remained significantly above baseline until week 52 for group 1, week 12 for group 2 and week 3 for group 3 (Figure 3b).

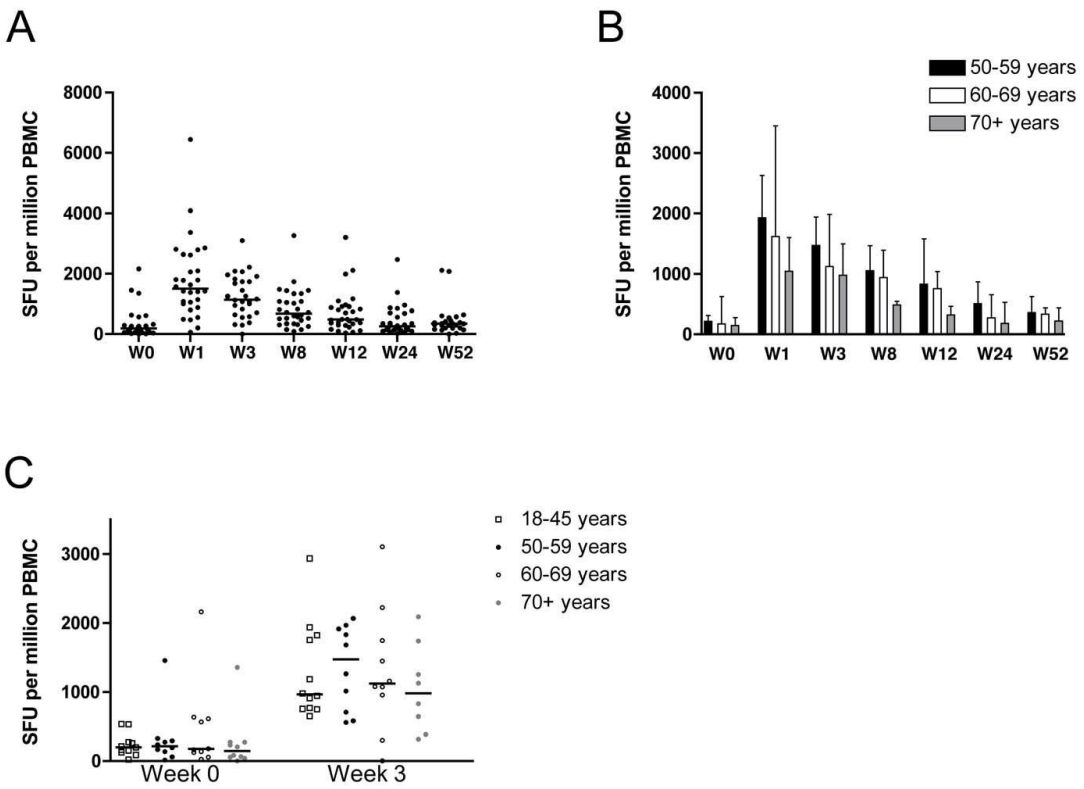


Figure 3. Ex vivo IFN-γ ELISpot responses to the vaccine insert. (A) Median and individual ex vivo IFN-γ ELISpot responses from vaccinated volunteers at baseline (week 0), and weeks 1, 3, 8, 12, 24, and 52. Significant differences between the pre- and post-vaccination time points were detected using the Wilcoxon signed rank test: week 1 ($p = 0.0001$), week 3 ($p = 0.0001$), week 8 ($p = 0.0001$), and week 12 ($p = 0.001$). (B) Median *ex vivo* IFN-γ ELISpot responses to the NP+M1 insert stratified according to age: black bars = group 1 (50–59 years), white bars = group 2 (60–69 years), and grey bars = group 3 (70+ years). Error bars indicate interquartile ranges. Significant differences between the pre- and post-vaccination time points were detected using the Wilcoxon signed rank test as follows. Group 1: week 1 ($p = 0.002$), week 3 ($p = 0.002$), week 8 ($p = 0.002$), week 12($p = 0.039$), week 24 ($p = 0.002$), and week 52 ($p = 0.0039$). Group 2: week 1 ($p = 0.002$), week 3 ($p = 0.002$), week 8 ($p = 0.002$), and week 12 ($p = 0.0371$). Group 3: week 1 ($p = 0.0039$) and week 3 ($p = 0.0195$). Significant differences were also detected between groups using the Mann- Whitney U-test, with responses in group 1 being higher than those in group 3 at week 3 ($p = 0.043$) and week 8 ($p = 0.023$). (C) Median and individual *ex vivo* IFN-γ ELISpot responses at week 1 and week 3 stratified according to age, and including a vaccinated cohort of younger (18–45 years) volunteers.

We previously vaccinated 15 healthy volunteers aged 18–45 years with MVA-NP+M1 using the same dose (1.5 x10^8 pfu) and route of administration (12). No significant differences in the ELISpot responses were detected between the younger volunteers (18–45 years) and the older volunteers (50+ years) either before or after vaccination (Figure 3c), although there was a trend towards higher responses in groups 1 and 2, and lower responses in group 3.

Flow cytometry was used to determine antigen-specific cytokine production (IFN-γ, TNF-α, IL-2, IL-10 and IL-17), T cell degranulation (CD107a mobilization) and granzyme B expression. MVA-NP+M1 was shown to boost both CD4+ and CD8+ T cell responses, and significant increases in IFN-γ, IL-2 and TNF-α production were observed in both populations at week 1 and week 3 post-vaccination (Figure 4). A significant increase in CD107a mobilization was only detected for CD8+ T cells at week 3 (p=0.004).

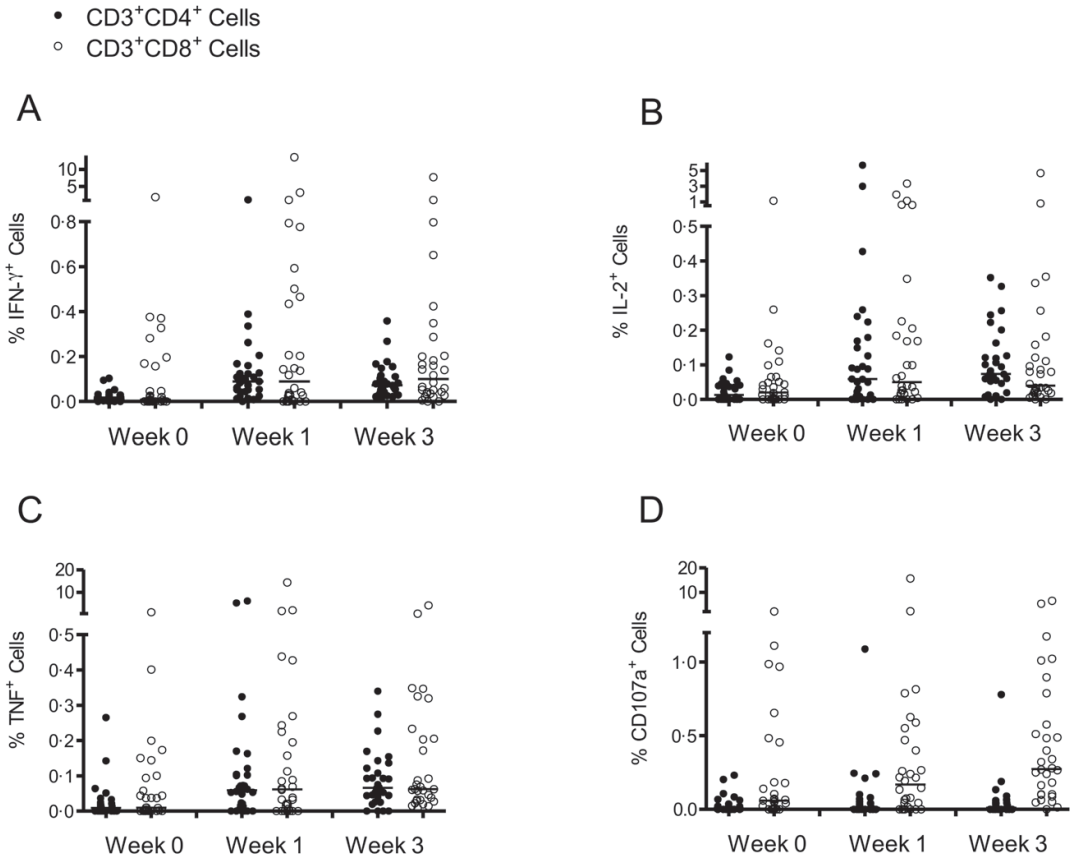


Figure 4. **IFN-γ, IL-2, TNF and CD107a responses to the vaccine insert measured by flow cytometry**. Production of IFN-γ (A), IL-2 (B) and TNF (C), and mobilization of CD107a (D), after background subtraction in $CD3^+CD4^+$ (black circles) and $CD3^+CD8^+$ (white circles) cell populations stimulated with a single pool of peptides spanning the complete NP+M1 vaccine insert. Volunteers in group 3 were tested at weeks 0, 1, and 3. Significant differences between pre- and post-vaccination time points were detected using the Wilcoxon signed rank test as follows: IFN-γ $CD4^+$, week 1 (p = 0.0001) and week 3 (p = 0.0001); IFN-γ $CD8^+$, week 1 (p = 0.001) and week 3 (p = 0.0005); IL-2 $CD4^+$, week 1 (p = 0.001) and week 3(p = 0.0001); IL-2 $CD8^+$, week 1 (p = 0.006) and week 3 (p = 0.03); TNF $CD4^+$, week 1 (p = 0.002) and week 3 (p = 0.0003); TNF $CD8^+$, week 1 (p = 0.0003) and week 3 (p = .?002); CD107a $CD8^+$, week 3 (p = 0.004)

No increase in the antigen-specific production of IL-10, IL-17 or granzyme B were detected in either the $CD4^+$ or $CD8^+$ T cell populations following vaccination (data not shown). However, as reported previously (17) the production of granzyme B from unstimulated $CD8^+$ T cells was significantly elevated at week 1 in the oldest age group (median = 57.8% of $CD8^+$ T cells) compared to group 1 (median = 27.1%, p = 0.002) and group 2 (median = 29.3%, p = 0.0115). Non-specific granzyme B production by $CD8^+$ T cells was also significantly elevated in group 3 compared to group 1 at week 3 (group 3 = 45,9%, group 1 = 25.2%, p = 0.0113).

Figure 5 shows the frequency of polyfunctional $CD4^+$ and $CD8^+$ T cells detected by flow cytometry using panel 1. The percentage of T cells with quadruple, triple and double functional outputs detected in the $CD4^+$ and $CD8^+$ populations increased significantly at week 1 and week 3 post-vaccination in group 1 (50–59 years). However, in groups 2 and 3, only the triple and double functional cells in the $CD4^+$ T cell population increased at the same time points. In group 3, a significant increase in quadruple and triple functional cells in the $CD8^+$ T cell population was detected at week 3 post-vaccination. The respective P values for these comparisons (Wilcoxon signed rank test) are shown in Table S2.

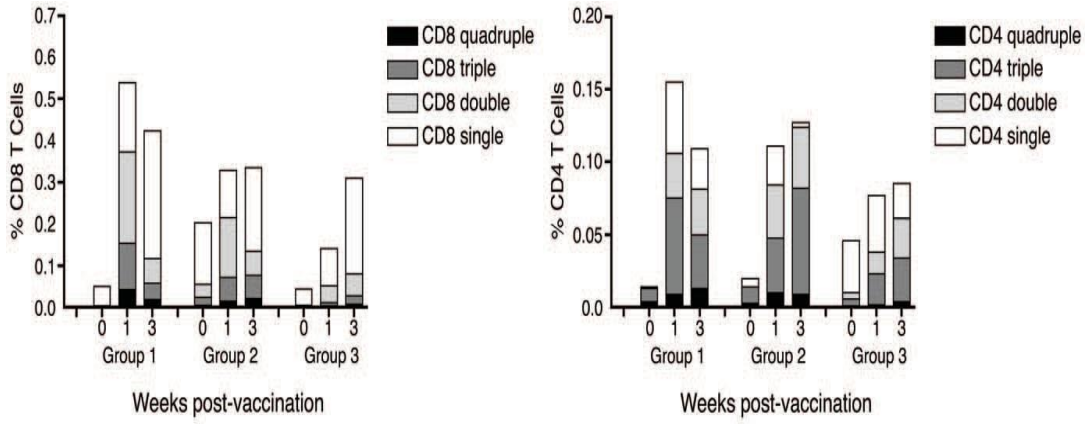
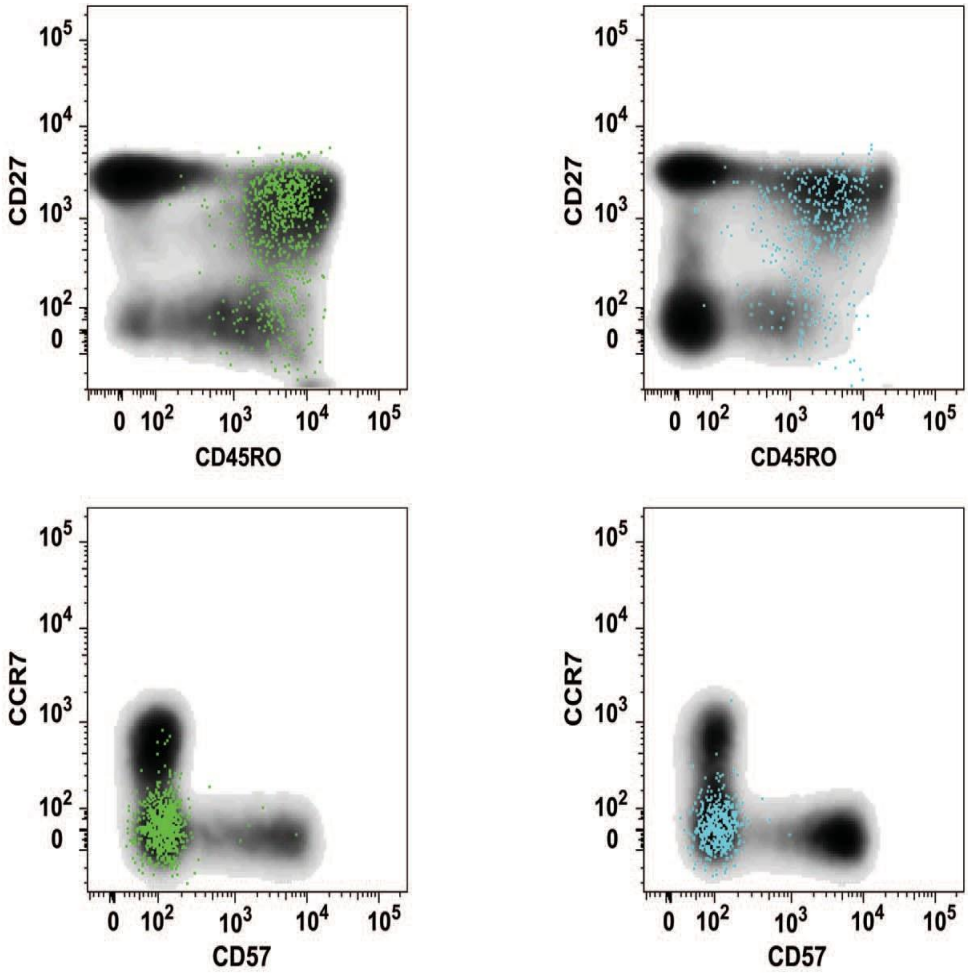


Figure 5. Functional profile of T cell responses to the vaccine insert measured by flow cytometry.
Mobilization of CD107a and production of IFN-c, IL-2 and TNF after background subtraction in $CD3^+CD4^+$ (A) and $CD3^+CD8^+$ (B) cell populations stimulated with a single pool of peptides spanning the complete NP+M1 vaccine insert. Median percentages of quadruple (black), triple (dark grey), double (light grey) and single (white) functional cells are shown.

In further analyses, we examined the clonotypic composition of $CD8^+$ T cells specific for the HLA A*0201-restricted GILGFVFTL epitope (M1, residues 58–66) using a template-switch anchored RT-PCR to amplify all expressed *TRB* gene products. A profound type IV bias was observed in these antigen- specific $CD8^+$ T cell populations, comprising strict TRBV19 usage combined with a central XRSX motif in the CDR3 loop (Figure 6) (18), consistent with previous reports (19,20). To determine the origins of these MVA-NP+M1 vaccine-expanded clonotypes, we conducted similar studies in a separate cohort of volunteers. These volunteers were aged 20–50 and had been vaccinated with either 5×10^7 pfu intradermally or 2.5×10^8 pfu intramuscularly (11). Paired samples from day 0 (pre-vaccination) and day 7 (post- vaccination) were available for three volunteers. In all cases, the dominant clonotypes were identical at both time points, indicating the expansion of pre-existing M1-specific memory $CD8^+$ T cells (Figure 7). However, the post-vaccination repertoires were more polyclonal due to the presence of less frequent clonotypes in greater numbers. This could reflect either *de novo* recruitment from the naïve pool or the expansion of clonotypes from the memory pool with pre-vaccination frequencies below the limit of detection. Notably, all sorted M1-specific $CD8^+$ T cell populations displayed a predominant $CD27^+CD45RO^+CD57^-CCR7^-$ memory phenotype (Figure 6 and data not shown). This phenotypic homogeneity is consistent with the functional homogeneity observed within the $CD8^+$ compartment before and after vaccination.

A



B

TRBV	CDR3	TRBJ	Freq (%)	Count
19	CASSDIALGEQY	2-7	15.28	11
19	CASSMRSSSEAF	1-1	11.11	8
19	CASSIRSSYEQY	2-7	9.72	7
19	CASSGRAGTEAF	1-1	9.72	7
19	CASSSLSNQPQH	1-5	8.33	6
7-4	CASSSLRGWPYEQY	2-7	6.94	5
19	CASSMRSSYEQY	2-7	6.94	5
19	CASSIRSTGELF	2-2	6.94	5
19	CASSIRSQETQY	2-5	4.17	3
19	CASSIRSTDTQY	2-3	4.17	3
19	CASSIHSNQPQH	1-5	4.17	3
19	CASSDRSNTEAF	1-1	4.17	3
19	CASSIRASYEQY	2-7	2.78	2
19	CASSTRSTGELF	2-2	2.78	2
19	CASSQRSTDTQY	2-3	1.39	1
19	CASCIHSNQPQH	1-5	1.39	1

TRBV	CDR3	TRBJ	Freq (%)	Count
19	CASSPRSTDTQY	2-3	44.58	37
19	CASSIRSAYEQY	2-7	21.69	18
19	CASSIRSSYEQY	2-7	14.46	12
19	CASSTRASYEQY	2-7	12.05	10
2	CASSEGGRSYNEQF	2-1	1.20	1
2	CASGEGGRSYNEQF	2-1	1.20	1
27	CASSRTLSTDTQY	2-3	1.20	1
19	CASMGLAGLNEQF	2-1	1.20	1
19	CTSSTRASYEQY	2-7	1.20	1
19	CASSPRSTDKQY	2-3	1.20	1

Figure 6. Phenotypic and clonotypic properties of M1-specific CD8$^+$ T cells elicited by MVA-NP+M1. (A) Phenotype of vaccine-elicited CD8$^+$ T cells specific for the HLA A*0201-restricted M1-derived epitope GILGFVFTL (residues 58–66). Antigen-specific CD3$^+$CD8$^+$tetramer$^+$ cells are shown as coloured dots superimposed on bivariate plots showing the phenotypic distribution of the total CD8$^+$ T cell population (grey density plots). Response sizes were 1.48% (left panels) and 0.75% (right panels) with respect to the total CD8$^+$ T cell population. (B) TRBV and TRBJ usage, CDR3 amino acid sequence and relative frequency of the GILGFVFTL-specific CD8$^+$ T cell clonotypes contained within the antigen-specific populations depicted in (A). Public clonotypes within the present dataset are colour-coded. Representative analyses are shown for volunteers in group 3 (70+ years).

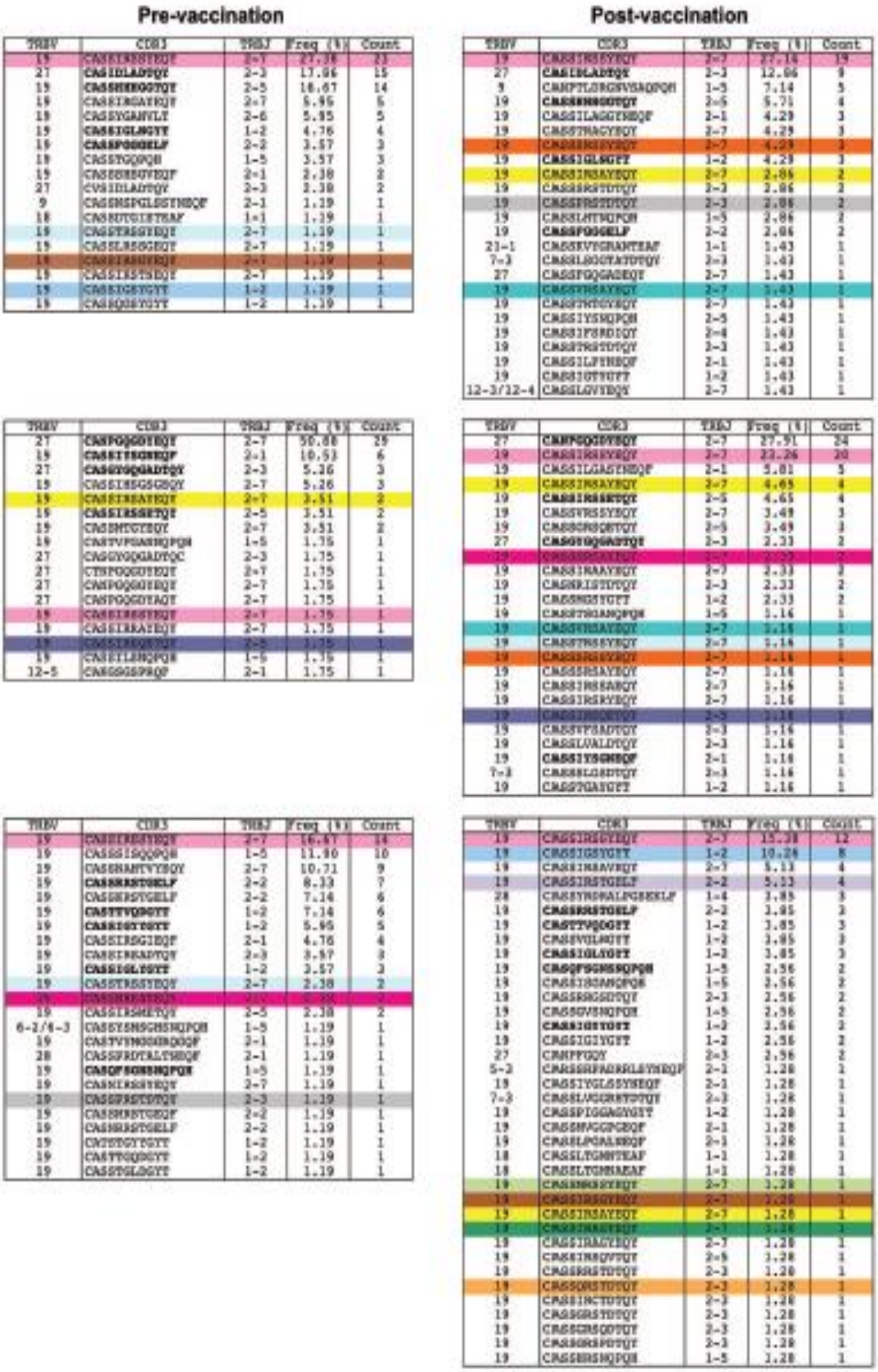


Figure 7. Patterns of clonotype usage in M1-specific CD8$^+$ T cell populations before and after vaccination with MVA-NP+M1. TRBV and TRBJ usage, CDR3 amino acid sequence and relative frequency are shown for GILGFVFTL-specific CD8$^+$ T cell clonotypes on day 0 (pre- vaccination) and day 7 (post-vaccination). Public clonotypes within the present dataset are colour-coded. Non-public clonotypes present at both time points within an individual are highlighted in bold type.

DISCUSSION

Here, we report the ability of MVA-NP+M1 to boost influenza- specific T cell responses in older adults. Recombinant MVA vaccines are establishing a good reputation for safety, although the majority of these data relate to younger individuals aged between 18–45 years. Our results with MVA-NP+M1 add to the experience from cancer trials with MVA-5T4 that recombinant MVA is safe in older adults (21). Indeed, no severe or serious adverse reactions were detected in our volunteers.

We also report that MVA-NP+M1 is highly immunogenic in volunteers over the age of 50 years. In one quantitative review (4) of trivalent inactivated influenza vaccines, rates of seroprotection and seroconversion among those over 60 years old were four times lower for H1 and B antigens, and twice as low for H3 antigens. In addition, although not powered to detect declining efficacy with age, an age stratification suggested a far lower efficacy rate for those over 70 years (4). Indeed, other studies have suggested that vaccine efficacy appears to be as low as 30–40% in this age group (22). On an individual level, declines in immunological function are unlikely to occur in a linear fashion (chronological age being only a surrogate indicator of biological age) (23). However, on a population level, declines in vaccine responsiveness are likely to be observed as average age increases. Indeed, in the oldest age group (70+ years), we observed a reduction in immunogenicity as detected by ex vivo IFN-γ ELISpot compared to the youngest age group (50–59 years), with significantly lower responses at 3 and 8 weeks post-vaccination. However, when the 3 age groups were compared to a younger cohort of volunteers (18–45 years) who received the same dose of the MVA-NP+M1 vaccine, no significant differences were detected.

The functional characteristics of the cellular responses produced by vaccination are potentially as important as magnitude (24). Subsets of CD4+ and CD8+ T-cells following vaccination with MVA-NP+M1 are capable of secreting both TNF and IL-2, in addition to IFN-γ. Increases in the number of such polyfunctional T-cells have been associated with protective immunity in some models of infection (25). We show here that MVA-NP+M1 vaccination can also induce polyfunctional CD4+ and CD8+ responses in older adults, as determined by flow cytometric assessment of CD107a mobilization and the production of IFN-γ TNF-α and IL-2.

MVA-NP+M1 is designed to expand T cells that are already present in the memory pool rather than prime naïve T cells *de novo*. Direct evidence for this mode of action comes from our comparison herein of M1-specific TCR sequences before and after vaccination.

This provides a biological rationale for the use of MVA-NP+M1 in elderly individuals due to the impairment of thymic output with age. The absolute number of NP- and M1-specific T cells required for host defence against influenza is not known. However, the median *ex vivo* IFN-γ ELISPOT response observed in the older volunteers peaked one week after vaccination at 1,603 SFU/million PBMC, which represents an 8.5-fold increase compared to the pre-vaccination response.

No vaccine-induced expression of granzyme B, IL-10 or IL-17 was detected in our cohort of older volunteers. However, we did detect significantly higher non-specific levels of granzyme B expression in group 3 (70+ years) compared to group 1 (50–59 years) at weeks 1 and 3 post-vaccination. It has been shown previously that baseline granzyme B expression in $CD8^+$ T cells is higher in ageing volunteers and that these cells are associated with a decreased ability to respond to stimulation with whole influenza virus (17). Degranulation and extracellular release of granzyme B can also cause inflammation and extracellular granzyme B has been implicated in increasing the risk of serious illness in the elderly, including the risk of influenza induced cardiovascular complications [26,27].

A high IFN-γ : IL-10 ratio may be associated with protection from influenza (28). The median frequency of NP- and M1- specific T cells that secreted IL-10 was low (below 0.006%) and did not increase after vaccination, whereas there was a significant increase in the number IFN-γ-secreting T cells following vaccination.

The memory phenotype of vaccine-induced $CD8^+$ T cell populations, at least for a subset of M1-specific cells, was remarkably similar to that observed pre-vaccination. Indeed, a marginal decrease in CD27 expression consistent with progressive differentiation post-vaccination was the only detectable change between the time points studied within individual volunteers (data not shown). Thus, minimal differentiation-associated functional variations would be expected. Interestingly, despite vaccine- mediated expansion of pre-existing memory clonotypes, the observed $CD27^+CD45RO^+CD57^-CCR7^-$ phenotype indicates a lack of terminal differentiation and senescence (29). This is encouraging from the perspective that durable T cell immunity may be feasible using this approach of boosting existing T cell memory with an MVA-vectored vaccine.

MVA-vectored vaccines have the advantage that they can be produced on the large scale required for widespread human vaccination. The low level of polymorphism in NP

and M1 across influenza A strains means that a vaccine such as MVA-NP+M1 could provide T cell mediated protection against all influenza A subtypes.

In summary, we have shown that the novel influenza vaccine candidate MVA-NP+M1 is safe and highly immunogenic in adults over 50 years old. Both $CD4^+$ and $CD8^+$ memory T cell responses are boosted, and have the capacity to secrete multiple cytokines. Indeed, despite the apparent reduction in immune responsiveness observed in the oldest volunteers in this study, there was still a significant induction of IFN-γ secreting cells and a significant increase in the proportion of $CD4^+$ and $CD8^+$ T cells capable of triple cytokine production after vaccination. These enhanced T cell responses could provide heterosubtypic T cell based immunity against influenza in the elderly.

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8.3 Paper 3 - Preliminary Assessment of the Efficacy of a T-Cell-Based Influenza Vaccine, MVA-NP+M1 in Humans

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Background. The novel influenza vaccine MVA-NP+M1 is designed to boost cross-reactive T-cell responses to internal antigens of the influenza A virus that are conserved across all subtypes, providing protection against both influenza disease and virus shedding against all influenza A viruses. Following a phase 1 clinical study that demonstrated vaccine safety and immunogenicity, a phase 2a vaccination and influenza challenge study has been conducted in healthy adult volunteers.

Methods. Volunteers with no measurable serum antibodies to influenza A/Wisconsin/67/2005 received either a single vaccination with MVA-NP+M1 or no vaccination. T-cell responses to the vaccine antigens were measured at enrollment and again prior to virus challenge. All volunteers underwent intranasal administration of influenza A/Wisconsin/67/2005 while in a quarantine unit and were monitored for symptoms of influenza disease and virus shedding.

Results. Volunteers had a significantly increased T-cell response to the vaccine antigens following a single dose of the vaccine, with an increase in cytolytic effector molecules. Intranasal influenza challenge was undertaken without safety issues. Two of 11 vaccinees and 5 of 11 control subjects developed laboratory-confirmed influenza (symptoms plus virus shedding). Symptoms of influenza were less pronounced in the vaccinees and there was a significant reduction in the number of days of virus shedding in those vaccinees who developed influenza (mean, 1.09 days in controls, 0.45 days in vaccinees, $P = .036$).

Conclusions. This study provides the first demonstration of clinical efficacy of a T-cell–based influenza vaccine and indicates that further clinical development should be undertaken.

A recent meta-analysis of influenza vaccine efficacy and effectiveness (1) concluded that protection against virologically confirmed influenza is at best moderate, and in some seasons is greatly reduced or completely absent. Even in the most favorable situation when the vaccine is exceptionally well matched to the circulating virus, as was the case for pandemic H1N1 vaccines, median effectiveness in adults <65 years was 69%. The size of the influenza vaccine market was US$2.8 billion in 2008–2009 in 7 major markets (United States, Japan, France, Germany, Italy, Spain, and United Kingdom) (2). An increasingly greater proportion of the population is vaccinated, with vaccination for all individuals aged >6 months recommended in some countries, but vaccines with considerably improved and more consistent effectiveness are required in order to bring about a greater reduction in influenza-related morbidity and mortality.

Trivalent inactivated vaccines are used as influenza vaccines in most circumstances, with live attenuated influenza vaccines sometimes used in children. Although cytotoxic T-cell–mediated immunity against influenza is an important component of naturally acquired immunity(3,4), the trivalent inactivated vaccine does not stimulate this response, and live attenuated influenza vaccine has been found to prime a T-cell–mediated response in young children but not to boost it in adults who have already acquired T-cell responses to influenza antigens following natural exposure to the virus(5). Because the main targets of T-cell recognition are internal antigens of the influenza virus that are well conserved between influenza A virus subtypes, (6) T-cell–mediated immunity should provide much broader protection than antibodies specific for the highly polymorphic external glycoproteins of the virus.

We have previously reported on the use of a novel influenza vaccine, to boost these cross-reactive T-cell responses in adult volunteers, in a phase I study that demonstrated the safety and immunogenicity of the vaccine (7). MVA-NP+M1 is a modified vaccinia virus Ankara (MVA) vector (replication-deficient) expressing the conserved internal influenza antigens nucleoprotein (NP) and matrix protein 1 (M1). T-cell responses to these antigens are known to be induced by influenza infection (6). We now describe a phase 2a vaccination and influenza challenge study, the first study to test the efficacy of an influenza vaccine designed to boost T-cell responses without inducing antihemagglutinin antibodies. The study confirmed vaccine safety and immunogenicity and provides preliminary evidence of vaccine

efficacy, with a 60% reduction of laboratory-confirmed influenza in vaccinated subjects.

MATERIALS AND METHODS

Vaccine Design and Manufacture. MVA-NP+M1 design and manufacture are described in (7).

Study Population. Volunteers were recruited and enrolled following written informed consent under a protocol approved by the UK Medicines and Healthcare Products Regulatory Agency and the Oxfordshire NHS Research Ethics Committee. Recruitment took place at the Centre for Clinical Vaccinology and Tropical Medicine, Oxford and the Welcome Trust Clinical Research Facility, Southampton. Volunteers were aged 18–45 years and were initially screened by haemagglutination inhibition (HI) assay against the virus to be used in the challenge phase of the study to ensure susceptibility to challenge. Those with a titre ≤1:10 were eligible for further screening. Enrolled volunteers were seronegative for human immunodeficiency virus (HIV), hepatitis B virus, and hepatitis C virus and had not received seasonal influenza vaccination for at least 1 year prior to enrollment. Results of routine hematological and biochemical tests on enrolled volunteers were all within normal limits.

Vaccination and Follow-up Regimen. Following receipt of study information, volunteers attended a screening visit to assess their suitability for the study. Two volunteers were screened, vaccinated, and underwent influenza challenge ahead of the main cohort. Subsequent eligible volunteers were enrolled first into the vaccination group and subsequently into the control challenge group. The volunteers taking part in the main efficacy cohort were screened for enrollment between 3 August 2009 and 9 September 2009. Influenza transmission rates during this period were low in the areas in which volunteers were recruited, and no volunteer had experienced an influenza-like illness prior to screening. Vaccinated volunteers received a single intramuscular injection of 1.5×10^8 plaque-forming units (PFUs) of MVA-NP+M1 (dose volume, 1154 μL) 28 days prior to entry to the quarantine unit. Volunteers were

reviewed on day 2 after vaccination to assess adverse events and on day 21 for exploratory immunology blood sampling.

Ex Vivo Interferon γ Enzyme-Linked Immunosorbent Spot Assay. The ex vivo interferon γ (IFN-γ) enzyme-linked immunosorbent spot assay (ELISpot) was performed as previously described [7]. Fifteen- to 20-mer peptides overlapping by 10 amino acid residues, spanning the whole of the NP + M1 insert in pools of 10 peptides, were used to stimulate peripheral blood mononuclear cells (PBMCs) at a concentration of 10 μg/mL. Fifty microliters of PBMCs (2×10^5 cells) and 50 μL of the peptides was tested in triplicate. R10 was used as a negative control, and phytohemagglutinin at a final concentration of 10 μg/mL was used as a positive control. Following an 18–20-hour incubation at 37°C, the ELISpot plates were developed, dried, and read with an AID ELISpot reader (AID Diagnostika). The results are expressed as spot-forming units (SFUs) per million PBMCs after background subtraction.

Flow cytometry, quarantine, and challenge procedures are described in the Supplementary Data.

RESULTS

Vaccine Safety and Immunogenicity. A total of 15 volunteers (11 for the main study, 2 for a pilot challenge study, and 2 volunteers who were vaccinated but then excluded from the influenza challenge for either increase in HI titre to the challenge virus or evidence of recent mild respiratory tract infection) were administered 1.5×10^8 PFU MVA-NP+M1 intramuscularly. The study timeline is shown in Supplementary Figure 1*A*. Supplementary Figure 1*B* shows the numbers of volunteers recruited for the vaccine and control groups, with demographic information given in Supplementary Table 1. The safety profile was comparable to other MVA- vectored vaccines, with the majority of adverse events being mild in severity. No severe systemic adverse events were reported.

T-cell responses to the influenza antigens NP and M1 were measured in all volunteers at screening and again on day of vaccination and 21 days later in the vaccinees and in all volunteers on the day prior to challenge, as well as 8 occasions after influenza challenge (Figure 1). As previously observed (7), there was a clearly detectable response in all volunteers at the time of screening, with a median

response of 258 SFUs per million PBMCs in the group who went on to receive the vaccine and 300 SFUs per million PBMCs in the controls. The level of response was stable prior to vaccination, significantly boosted to 980 SFUs per million PBMCs 21 days after vaccination ($P < .001$ vs day 0) and then declined to 627 SFUs per million PBMCs 8 days later (the day prior to influenza challenge, $P < .05$ vs day 0). The response in the control group remained stable prior to influenza challenge, with a median of 215 SFUs per million PBMCs measured on the day prior to influenza challenge (day 29). Although there was no significant difference in the responses between the 2 groups at screening, responses to NP and M1 at day 29 were significantly higher in the vaccinees compared to controls ($P < .05$) (Figure 1).

The response to all influenza antigens in addition to those included in the vaccine was also measured on day 29 using overlapping peptides for each antigen (Supplementary Figure 2). The only statistically significant difference between responses in vaccinees and controls was the magnitude of the response to the vaccine antigens, with the response to NP predominating in most vaccinees.

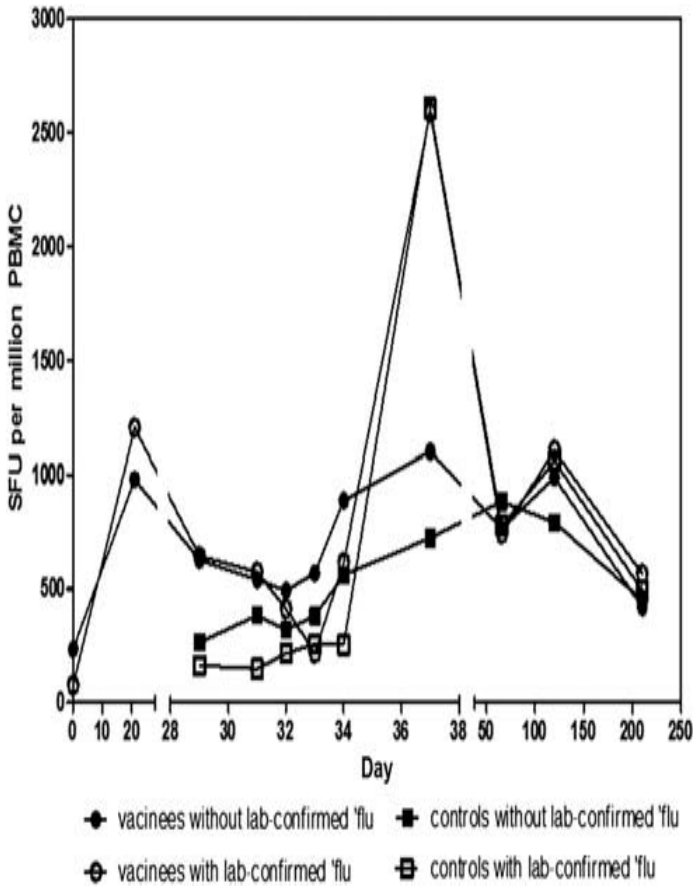


Figure 1. Ex vivo interferon γ enzyme-linked immunosorbent spot assay responses to nucleoprotein (NP) and matrix protein 1 (M1). The graph represents the summed response to NP and M1 antigens in vaccinees (circles) and controls (squares) at the relevant time points; lines represent the median per group and open symbols represent subjects who developed laboratory-confirmed influenza. Control subjects were not assayed at day 0 or day 21. Vaccination took place on day 0 and influenza challenge on day 30. Data were analyzed with a Kruskal-Wallis 1-way analysis of variance with selected pairs of data analyzed with a Dunn positive test. No significant difference between the median response in the vaccinated and control group was observed at time of screening (day 0 for vaccinees, day 29 for controls). A significant increase in the response was observed in vaccinees between days 0 and 21 and days 0 and 29 ($P < .001$, $P < .05$, respectively). A significant difference between vaccinees and controls was observed at day 29 ($P < .05$). Abbreviations: PBMC, peripheral blood mononuclear cell; SFU, spot-forming units

T-Cell Phenotype of the Immunodominant Response to M158–66 in Vaccinated and Control Volunteers. Six of the vaccinees and 7 of the controls were positive for human leukocyte antigen A*0201 and therefore likely to have pre-existing T-cell responses to the known A2-restricted immunodominant epitope $M1_{58–66}$. A tetramer for this epitope was used to measure phenotypic markers in PBMCs from these volunteers. A significant difference between vaccinated and control donors on the day prior to influenza challenge was observed in the expression of the cytotoxic markers perforin (D48 epitope (8) and granzyme A (Figure 2), indicating that the antigen-specific $CD8^+$ T cells in the vaccinees were more highly activated than T cells from the control donors.

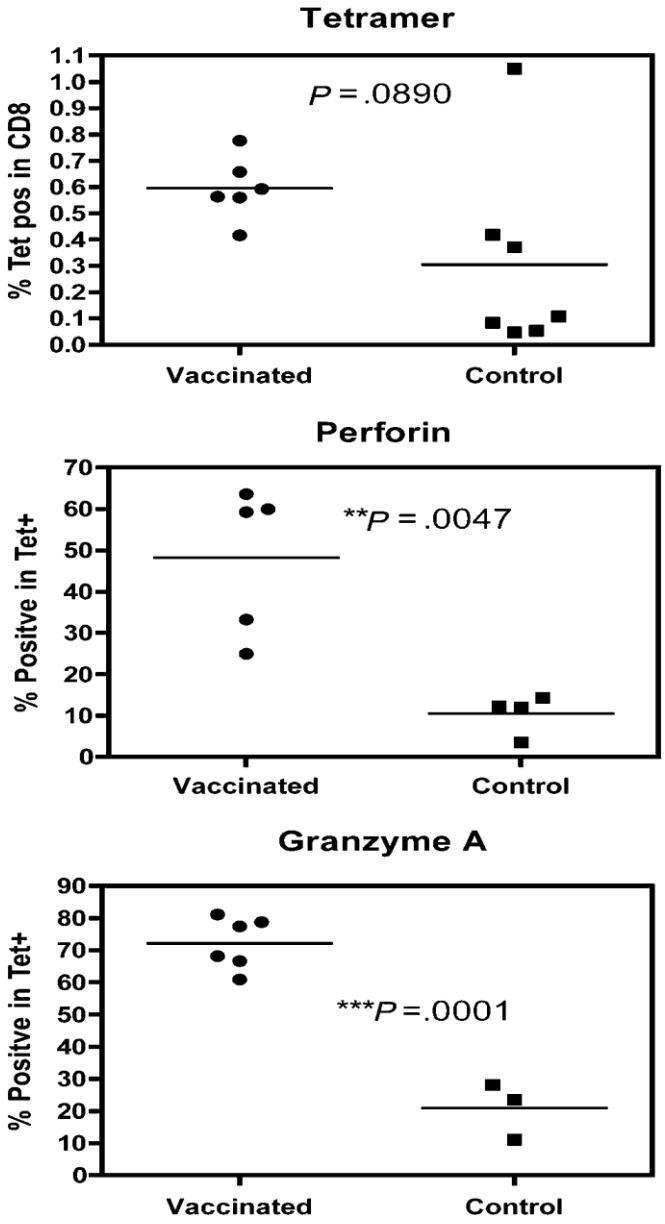


Figure 2. Responses to $M1_{58–66}$ in human leukocyte antigen A2–positive volunteers. Whole blood drawn 1 day prior to virus challenge was labeled for tetramer (A*0201/GILGFVFTL) followed by perforin or granzyme A staining. Values shown are the percentage of $CD8^+$ T cells or Tet^+ cells; individuals are shown as a single point with lines representing the median per group. Open symbols represent samples from volunteers who subsequently developed laboratory-confirmed influenza. For each marker the data were analyzed with an unpaired *t* test; *P* values are shown for statistically significant differences between vaccinees and controls.

Influenza Challenge Outcome. The safety of the influenza challenge protocol in healthy volunteers is well established, but as this was the first study to our knowledge in which T-cell responses to influenza antigens were boosted by vaccination prior to influenza challenge of human volunteers by intranasal administration, we conducted a pilot safety study of 2 vaccinated volunteers to make an initial assessment of the safety of the protocol prior to the main study. These 2 volunteers underwent the same screening, vaccination, quarantine, and challenge protocol as for the main study, including twice-daily symptom questionnaires and once-daily physician-directed examination to assess their response to influenza challenge following MVA-NP+M1 vaccination. The majority of symptoms recorded were mild, with some

evidence of upper respiratory tract infection. Rhinorrhea was the commonest symptom, but no cough or other symptoms of lower respiratory tract infection or severe illness were observed. Following safety review, permission was granted to proceed with influenza challenge for the main study of 11 vaccinees and 11 control subjects.

The primary outcome of the challenge study was the number of subjects in each group diagnosed with laboratory-confirmed influenza, defined as mild or moderate/severe symptoms of influenza infection plus laboratory detection of influenza virus in any of the daily nasal washes conducted following influenza challenge (Table 1). In total, 2 vaccinees and 5 controls developed laboratory-confirmed influenza. Of these, 1 vaccinee and 4 controls experienced moderate to severe symptoms in addition to virus shedding. Comparing the vaccinated and control groups as a whole, symptoms were fewer in vaccinees at all time points following influenza challenge (Figure 3*A*), with symptoms peaking on the second and third days. Vaccinees as a group experienced a significant reduction in the number of days of virus shedding in the presence of laboratory-confirmed influenza (5 of 55 days in vaccinees and 12 of 55 days in controls; $P = .036$).

Table 1. Clinical Outcome of Challenge

Vol No.	Total Symptom Score	Symptom Severity	Virus (Log_{10} $TCID_{50}$) Shed on Day After Challenge					Lab-Confirmed Influenza	HI Postchallenge
			1	2	3	4	5		
A: Vaccinees									
58	26	Mild						N	<10
76	3	None						N	20
79	0	None						N	40
80	0	None				1.75	3.00	N	<10
19	0	None						N	80
32	12	Mild						N	<10
37	27	Mild						N	160
39	29	Mod/sev	3.25	4.25				Y	>640
41	3	None						N	320
64	12	Mild			2.5	2.96	3.75	Y	226
70	0	None						N	>640
B: Controls									
72	29	Mod/sev						N	160
81	20	Mild		3.25	2.00			Y	<10
84	29	Mod/sev	2.75	3.25				Y	40
95	4	Mild		3.5				Y	<10
100	35	Mod/sev			3.5	5.5	1.75	Y	160
87	38	Mod/sev	3.00	3.25	2.5	1.75		Y	80
86	0	None						N	320
93	1	None						N	320
96	4	Mild						N	20
108	0	None						N	20
109	8	Mild						N	80

Laboratory-confirmed influenza is defined as mild or moderate to severe symptoms of influenza infection plus shedding of influenza virus on at least 1 day after challenge. Standardized nasal washes and virus assays were performed each day for 5 days on each subject, with no missing data points, but only positive results are shown in the table. The severity of the symptoms is defined by the symptom + examination score, with mild flu having a score of 4–28, moderate to severe is ≥29. HI titers were all <10 at screening and on entry to the quarantine unit. The figures given above are HI titers at study day 66 (26 days after influenza challenge) with the exception of volunteer 093 (study day 120, 90 days after influenza challenge).

Abbreviations: HI, hemagglutination titer; TCID50, median tissue culture infective dose

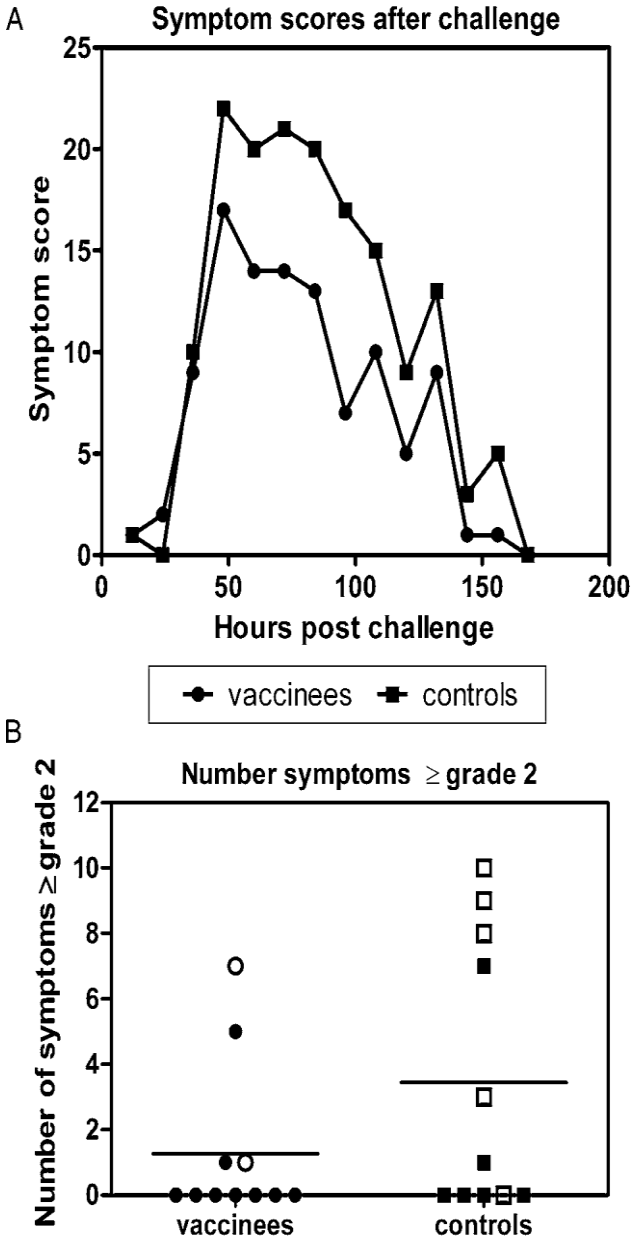


Figure 3. Total of symptom scores at each time point following challenge (*A*) or total grade 2 and 3 symptom and examination scores (*B*) for vaccinees (circles) and controls (squares), with the group mean indicated by a line. Open symbols denote subjects who developed laboratory- confirmed influenza after challenge.

Investigation of Immune Responses Associated With Protective Outcome. T cell responses to all influenza antigens in PBMCs on the day prior to influenza challenge by IFN-γ ELISpot assay were measured (Supplementary Figure 2). Following influenza challenge, there was no correlation between the total symptom score for each subject and the T-cell response to the vaccine antigens NP and M1, to all internal antigens (NP, M1, M2, NS1, NS2, PB1, PB2, PA) or to all influenza antigens (internal plus HA, NA) on the day prior to challenge. ELISpot assays were repeated on days 1, 2, 3, 4, and 7 after challenge (study days 31–37), measuring responses to NP and M1 only. There were

minor fluctuations in the number of PBMCs secreting IFN-γ in response to NP and M1 between days 29 and 34, with a pronounced increase on day 37 in those volunteers who developed laboratory-confirmed influenza (Figure 1). There was a significant positive correlation ($P = .0008$) between the total symptom score for each volunteer and the fold increase in ELISpot response from day 29 to day 37 (Supplementary Figure 3) when both control subjects and vaccinees are assessed.

Blood samples were also taken for ELISpot assay at follow-up visits on days 66, 120, and 210. The median response to NP and M1 declined between day 37 and day 66, marginally increased at day 120 (not significant) and decreased at day 210 in both vaccinated and control groups (Figure 1).

HI Titres After Challenge. HI titres to the challenge virus were repeated 36 days following influenza challenge (Table 1). There was no correlation between symptoms and virus shedding and rise in HI titre.

DISCUSSION

In this phase 2a study, we have demonstrated the safety of MVA-NP+M1 at a dose of 1.5×10^8 PFU given as a single intramuscular injection. The majority of adverse events were mild in severity, with no serious systemic adverse events and no rigors experienced by any of the 15 subjects who were vaccinated, indicating a satisfactory safety profile at this dose. This dose is now being tested in an additional phase I study of subjects aged >50 years.

In the phase I study, the T-cell response was measured by ex vivo IFN-γ ELISpot assay at the peak of response 7 days after vaccination, and at 21 days. Median responses were 2793 and 2088 SFUs per million PBMCs at 7 and 21 days in the high-dose (2.5×10^8 PFU) group when fresh PBMCs were used in the assay. In the phase 2a study reported here, employing an intermediate dose of MVA-NP+M1 and using fresh PBMCs, the median response of the vaccinees 21 days after vaccination was 980, falling to 627 on the day prior to influenza challenge. Although it is not unexpected that the response measured by this assay is reduced when the vaccine

dose is reduced, the small numbers of volunteers in both studies do not allow an accurate determination of the magnitude of this reduction.

Following influenza virus challenge, only 5 of 11 control subjects developed laboratory-confirmed influenza, defined as symptoms of influenza disease plus virus shedding. This figure is lower than expected for challenge studies of this type, although it has previously been shown that approximately one-third of individuals undergoing influenza challenge are protected despite not having detectable antibodies against the challenge virus (3)and it is a known feature of this challenge model that not all control subjects will develop influenza. In this study only 2 vaccinated volunteers developed laboratory-confirmed influenza, the total number of symptoms recorded was lower in the vaccinated group at all time points following challenge, the number of grade 2 and 3 symptoms recorded was lower, and virus shedding was significantly reduced, supporting a protective effect of the vaccine against both disease severity and virus shedding.

It was notable that there was no consistent rise in HI titre following influenza challenge, even among volunteers who developed laboratory-confirmed influenza.

Having demonstrated a significant increase in the number of T cells producing IFN-γ in response to NP and M1 following vaccination, and with fewer vaccinated volunteers developing influenza than control subjects, we attempted to confirm the association of vaccine-induced T-cell responses with this protective outcome. In a large study of 2172 children in the Philippines and Thailand, it was found that the majority of infants and young children with >100 SFUs per million PBMCs in an IFN-γ ELISpot assay utilizing whole influenza virus as antigen were protected against clinical influenza (9). In our own small-scale study of adults, who would have had multiple prior exposures to influenza prior to vaccination resulting in memory populations of influenza-specific T and B cells, we were not able to define a correlate of protection based on responses detected in PBMCs using the IFN-γ ELISpot assay prior to challenge. Following influenza challenge, only minor fluctuations in the IFN-γ ELISpot were detected for a period of 4 days, increasing by the seventh day in subjects who developed influenza disease, whereas virus shedding was detected on the second and third day. This suggests that changes in responses measured in circulating PBMCs are occurring only after respiratory tract symptoms, and cannot be used to predict protection or susceptibility. However an anamnestic mucosal T-

cell response predictive of protection cannot be excluded. For future studies, a systems biology approach should be taken to understanding multifactorial mechanisms of protection that may be missed when only a small number of measures of immune system status are used.

This study provides evidence that intranasal challenge with influenza virus appears safe in individuals with elevated T-cell responses after MVA-NP+N1 immunization. The absence of any lower respiratory symptoms or signs, together with normal oxygen saturations and spirometry after influenza challenge, makes immunopathology highly unlikely. This supports previous work in several nonhuman species (particularly mice and ferrets (10) and pigs (11), indicating the apparent safety of intranasal influenza virus challenge after immunization with T-cell–inducing vaccines.

This first efficacy study of a vaccine designed to boost T-cell responses to conserved influenza antigens has demonstrated the safety of this vaccination approach. Vaccinees were exposed to influenza virus at a time when anti-influenza T-cell responses had been increased by vaccination with no ill effects and no evidence of lower respiratory tract infection or inflammation. It also elucidated the efficacy of the vaccine in boosting the T-cell response to the vaccine antigens and in reducing laboratory-confirmed influenza in the vaccinees compared with control subjects. This reduction equates to 60% vaccine efficacy, which is a similar level to that shown for inactivated influenza vaccines when the circulating virus and the strain used in the vaccine are well matched (12), although further studies using a larger sample size will be required to reach a more precise and robust estimate of vaccine efficacy.

The majority of studies on T-cell–mediated protection against influenza have been conducted in the mouse model. A small number of studies in other species have indicated that T-cell responses to conserved influenza antigens can protect against disease and virus shedding (13-16) but this is the first clinical efficacy study of a vaccine designed to protect in this way. The results of this first clinical study are encouraging and provide initial evidence that this approach will be successful. Further studies are indicated to characterize safety and efficacy in larger numbers of individuals and to assess vaccine immunogenicity in both older and younger age groups.

SUPPLEMENTARY TEXT

Vaccine Safety. The safety profile was comparable to other MVA vectored vaccines, with the majority of adverse events being mild in severity. No severe systemic adverse events were reported. One volunteer reported severe local pain and swelling that limited arm movement, which resolved within 24 hours. The median duration of systemic adverse events was 1 day (range 1-6) with local events lasting a median of 2 days (range 1-7).

Flow cytometry. Lymphocytes were stained with the following antibodies: anti-CD28-FITC, anti-CD57-FITC, anti-CD45RA-FITC, anti-CD38-PE-Cy7, anti-CD45RO-PECy7, anti-CD8-PerCP (Biolegend), anti-CD4-QD655 (Invitrogen), anti-CD4-Pacific Blue (eBioscience), anti-CD14-Pacific Blue (Invitrogen), anti-CD19-Pacific Blue (Invitrogen), anti-CD27-APCH7, anti-HLA-DR-APC, anti-CD25-APC, anti-CCR5-APC, anti-CD8-APC-AF750 (eBioscience) and anti-CD3-AlexaFluor700 (eBioscience). Intracellular staining was performed using Perm Buffer II (Becton Dickinson) as per manufacturer's instruction with the following antibodies: anti-Perforin-FITC (D48, Genprobe), anti-Granzyme A-FITC, anti-Granzyme B-FITC or Ki67-FITC. All antibodies were from Becton Dickinson unless stated otherwise. Samples and appropriate controls were collected on a Cyan Cytometer (Dako) and were analysed using FlowJo (Treestar). Absolute numbers of CD4+, CD8+ and CD3+ cells were measured with BD Trucount according to manufacturer's instructions (Becton Dickinson).

Quarantine and challenge. In total, 12 vaccinees and 12 control subjects entered the quarantine facility together (study day 28), for further assessment prior to intranasal influenza challenge. One vaccinee was found to be displaying symptoms of recent mild upper respiratory tract infection, was isolated from the remainder of the volunteers for further assessment and subsequently excluded prior to influenza challenge. One control had an apparent rise in HI titre to the challenge virus, and was also excluded. Vaccinated and control volunteers underwent clinical examination, spirometry, safety blood tests and electrocardiography on entry to the quarantine facility. Two days after entry to quarantine (to allow time for observation of any symptoms of respiratory virus infections) vaccinated and control volunteers were challenged with intranasal administration of H3N2 influenza (A/Wisconsin/67/2005) at a dose of 1ml of $10^{5.25}$ TCID50/ml. All volunteers were

inoculated during the same two hour period. After challenge, volunteers were followed up in the quarantine unit, using Retroscreen protocols as previously described (160). Self-reported symptoms were collected twice daily and a physical examination by a physician blinded to vaccination status was carried out daily. Nasal lavage fluid (standardised volume) for quantification of viral shedding was obtained daily. Symptoms were recorded using a standardised scoring system with a modified Jackson score of ≥4 indicating mild influenza disease, and a score of ≥29 indicating moderate to severe influenza disease. Symptoms directly elicited were headache, muscle/joint pains, nasal symptoms, cough, fever, sore throat, malaise, nausea and any other symptoms. Symptoms were scored individually and then summed together with clinical examination findings to give an overall score. Exploratory immunology samples were obtained on the day prior to challenge and then on days 1 to 4 and day 7 post challenge. Safety blood tests and further spirometry and electrocardiography were performed on all volunteers whilst in quarantine. A five day course of oseltamivir was commenced from day 5 post challenge for all volunteers with medication provided to complete the course after discharge. Volunteers were released from quarantine on the 7^{th} day post challenge after a negative rapid antigen test for influenza on nasal washings was obtained. After discharge, all volunteers were followed up on days 66, 120 and 210 post vaccination for further exploratory immunology and safety blood tests.

$TCID_{50}$ assay for virus shedding. Nasal wash samples were titrated on MDCK cells and the end point identified by cytopathic changes, by laboratory staff blinded to vaccination status.

Table S1 demographics. Volunteers 5 and 26 (shaded grey) took part in the pilot safety challenge study. The remainder of the volunteers took part in the main challenge study. For these, the mean (and range) age of volunteers was 28.7 (19 – 45) years for vaccinees and 30.0 (21 – 43) years for controls, (difference of medians non-significant Mann-Whitney p= 0.60). Mean Body Mass Index was 22.4 (19.5 – 26.9) and 23.7 (19.6 – 28.9) kg/m^2 for vaccinees and controls (difference of medians non-significant Mann-Whitney p= 0.26). It is unlikely that any of the volunteers had previously received smallpox vaccination and prior receipt of an MVA-vectored vaccine was an exclusion criterion.

A vaccinees

Volunteer	Age	Gender	BMI
5	42	M	24.0
26	42	M	19.4
19	20	M	23.3
32	35	F	23.6
37	22	F	19.6
39	23	M	19.5
41	32	F	26.8
58	21	M	23.2
64	19	F	18.9
70	45	F	24.3
76	43	M	26.9
79	21	F	20.6
80	35	F	20.0

B controls

Volunteer	Age	Gender	BMI
72	43	F	23.6
81	43	F	28.9
84	35	M	21.9
86	18	F	24.0
87	21	F	19.6
93	24	M	20.8
95	37	M	24.4
96	23	F	24.5
100	32	M	23.1
108	28	M	29.1
109	26	F	21.1

FIGURE LEGENDS

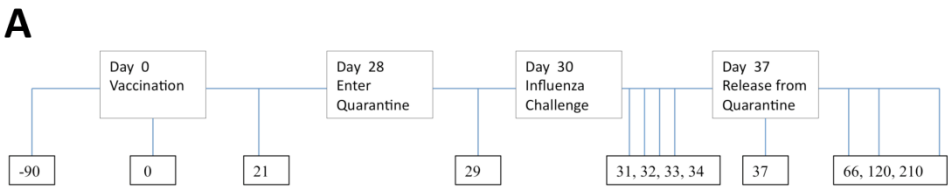
Figure S1 A Study timeline. Large boxes indicate major stages in the study, small boxes indicate blood sampling points.

Figure S1 B Recruitment Flow Chart

Figure S2 Ex vivo IFN-γ ELISpot responses to all influenza antigens. Summed $CD4^+$ and $CD8^+$ T cell responses to all influenza antigens measured by interferon-*γ* ELISpot assay using overlapping peptides to each antigen (20mers overlapping by 10), measured on study day 29, one day prior to influenza challenge, reported as spot forming units per million PBMC. The summed responses to external antigens are not significantly different between vaccinees and controls (Two-sample Wilcoxon rank-sum [Mann-Whitney] test P=0.0818) whereas summed responses to internal antigens are significantly different between the two groups if vaccine antigens are included (P=0.0003) but not when the vaccine antigens are excluded (P=0.818). For external influenza antigens, responses to H1, H3 and swine origin HI pandemic virus were tested.

Figure S3: Increase in T cell response following influenza challenge. A significant correlation between fold increase in ELISpot response (summed response to NP and M1) from day 29 to day 37, and the total symptom score was observed (Spearman r value = 0.6623, p= 0.0008 two-tailed) when vaccinees (circles) and control (squares) subjects were analysed together. Open symbols represent volunteers who subsequently developed laboratory confirmed influenza, closed symbols volunteers who did not.

Figure S1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Day 0
Vaccination
Day 28
Enter
Quarantine
Day 30
Influenza
Challenge
Day 37
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Quarantine
-90
0
21
29
31, 32, 33, 34
37
66, 120, 210

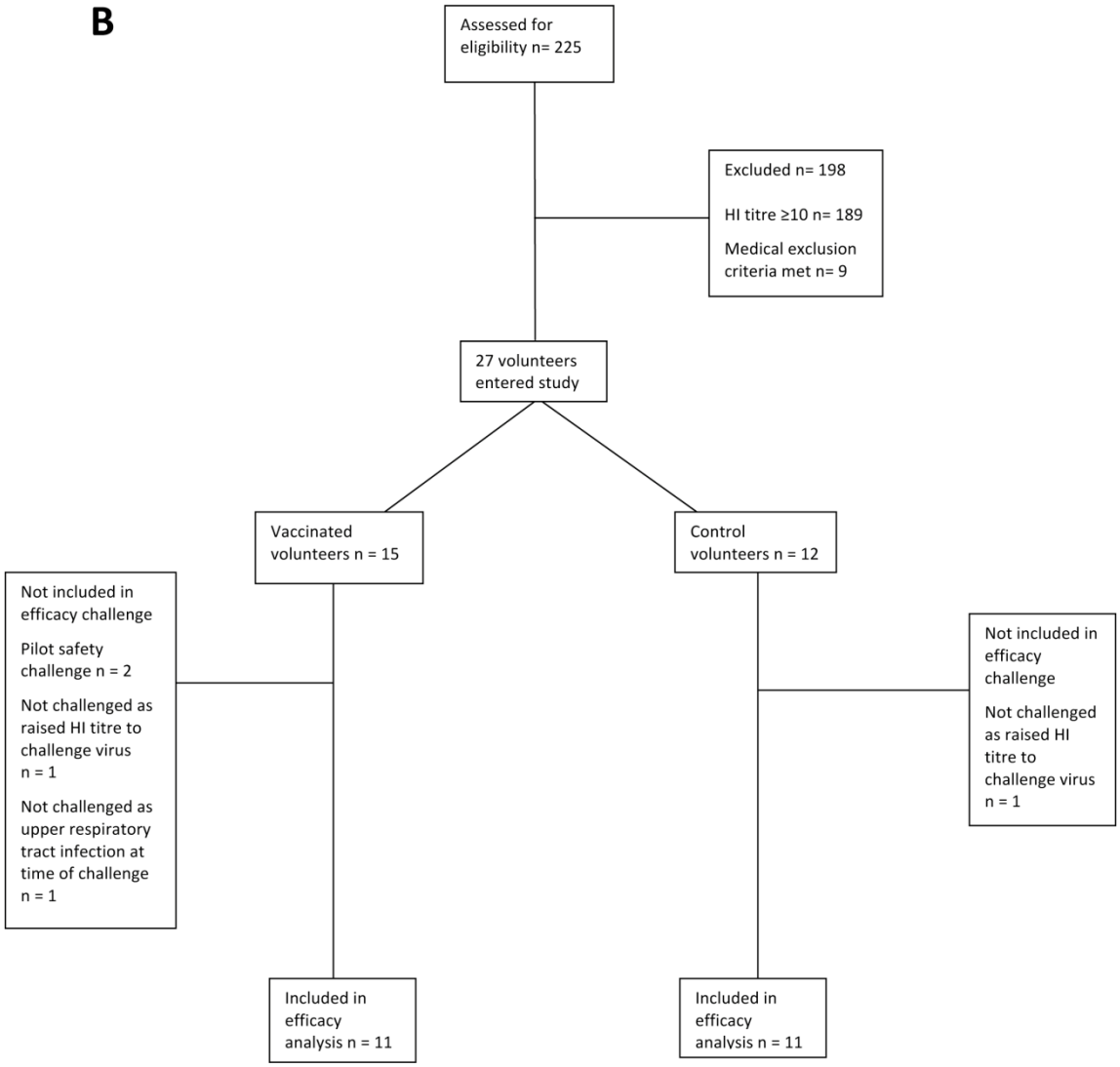
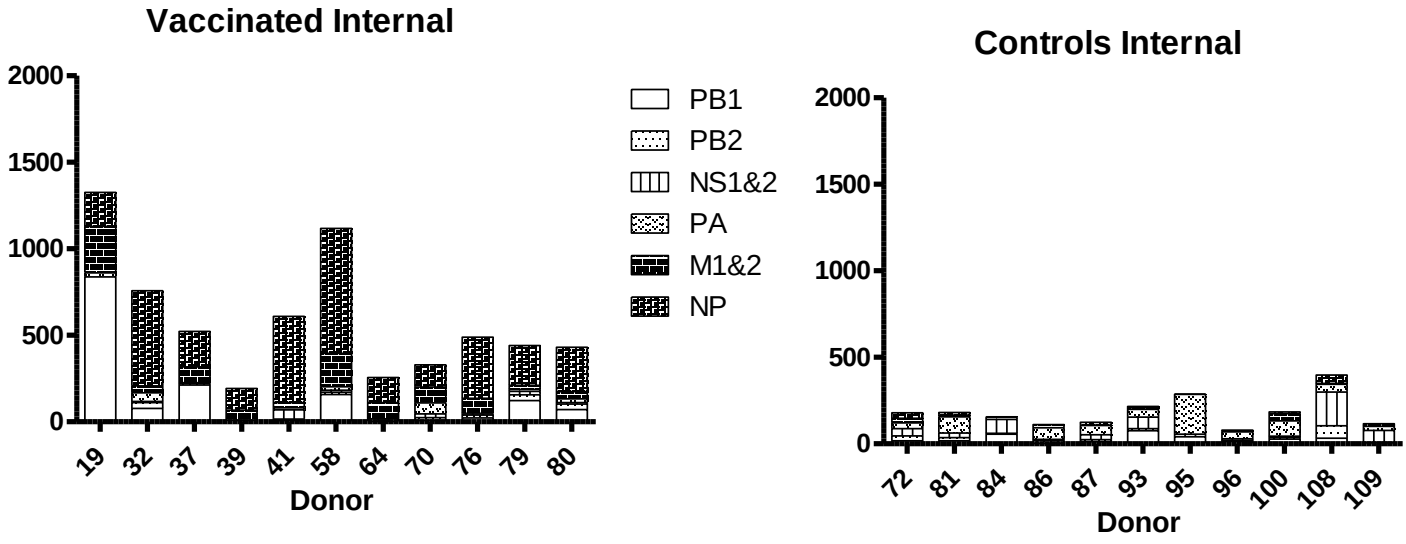

B
Assessed for eligibility n= 225
Excluded n= 198
HI titre ≥10 n= 189
Medical exclusion criteria met n= 9
27 volunteers entered study
Vaccinated volunteers n = 15
Control volunteers n = 12
Not included in efficacy challenge
Pilot safety challenge n = 2
Not challenged as raised HI titre to challenge virus n = 1
Not challenged as upper respiratory tract infection at time of challenge n = 1
Not included in efficacy challenge
Not challenged as raised HI titre to challenge virus n = 1
Included in efficacy analysis n = 11
Included in efficacy analysis n = 11

Figure S2


Vaccinated Internal
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1500
1000
500
0
19
32
37
39
41
58
64
70
76
79
80
Donor
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PB2
NS1&2
PA
M1&2
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72
81
84
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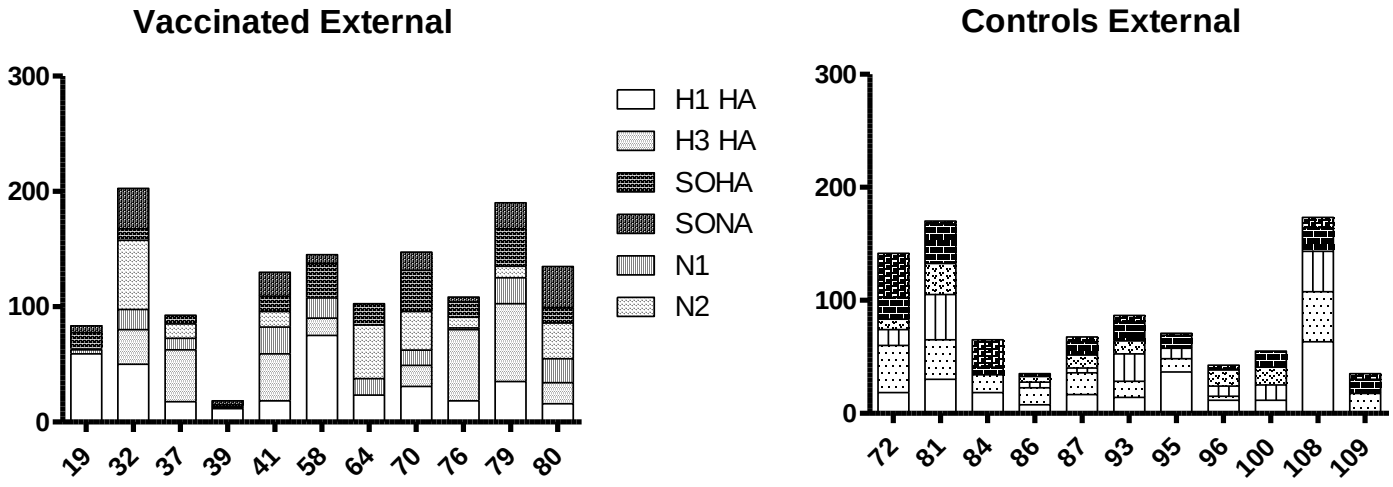
87
93
95
96
100
108
109
Donor


Vaccinated External
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200
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32
37
39
41
58
64
70
76
79
80
H1 HA
H3 HA
SOHA
SONA
N1
N2
Controls External
300
200
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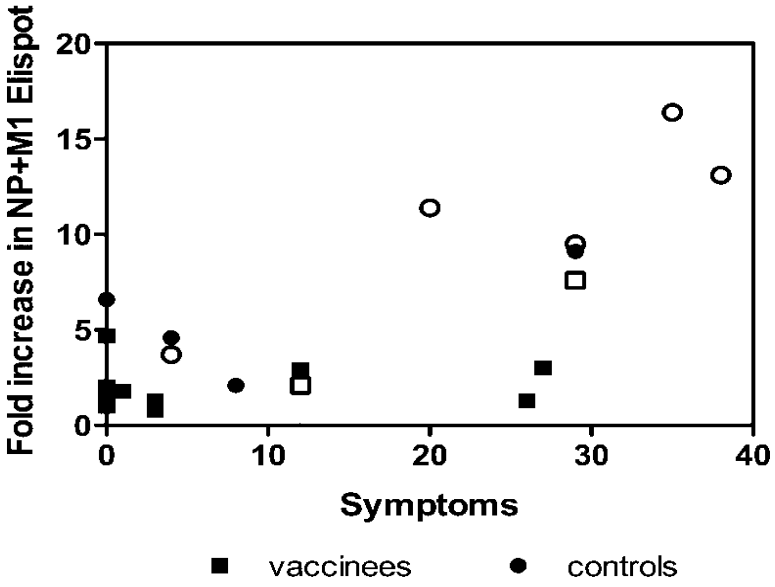
72
81
84
86
87
93
95
96
100
108
109

Figure S3


Fold increase in NP+M1 Elispot
20
15
10
5
0
0
10
20
30
40
Symptoms
vaccinees
controls

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8.4 Paper 4 - Examination of Influenza Specific T- Cell Responses after Influenza Virus Challenge in Individuals Vaccinated with MVA-NP+M1 Vaccine

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ABSTRACT

Current influenza vaccines stimulate neutralising antibody to the haemagglutinin antigen but as there is antigenic drift in HA it is difficult to prepare a vaccine in advance against an emergent strain. A potential strategy is to induce CD8+ and CD4+ T cells that recognize epitopes within internal proteins that are less subject to antigenic drift. Augmenting humoral responses to HA with T cell responses to more conserved antigens may result in a more broadly protective vaccine. In this study, we evaluate the quality of influenza specific T cell responses in a clinical trial using MVA-NP+M1 vaccination followed by influenza virus challenge. In vaccinated volunteers, the expression of Granzyme A, Perforin and CD57 on influenza HLA A*02 $M1_{58–66}$ antigen specific cells was higher than non-vaccinated volunteers before and after challenge despite a similar frequency of antigen specific cells. BCL2 expression was lower in vaccinated volunteers. These data indicate that antigen specific T cells are a useful additional measure for use in human vaccination or immunization studies.

INTRODUCTION

Protection against influenza virus requires antibody secretion by B cells and cytotoxic and soluble mechanisms mediated by T cells (1). The antibody response can be stimulated by vaccination and the existence of an influenza specific haemagglutination-inhibition antibody titre of 1:40 or more is associated with protection (2). Influenza virus undergoes antigenic shift and drift, generating novel influenza viruses to which people may not have immunity (3). One way of overcoming this lack of immunity could be to stimulate pre-existing cross-reactive CD4+ and CD8+ T cell responses which have been shown in humans to react with H1N1 2009 virus (4) and H5N1 (5,6). This heterosubtypic immunity is associated with protection during human experimental influenza infection (7). CD8+ T cell responses to one conserved A*02 matrix protein 1 $M1_{58–66}$ epitope can be protective in A2

transgenic mouse models (8) and are commonly found in healthy donors with this common HLA type (9). Therefore it is convenient to analyse $M1_{58–66}$ specific CD8+ T cells in vaccine studies. The phenotype or activation state of T cells is important for protection against influenza such that na¨ıve cells are less able to protect than activated or memory cells against a lethal influenza infection (10,11). Activated influenza specific T cells have been shown to be associated with protection against influenza in human studies (12,13,14,15) but no study of surface or intracellular phenotype was done.

We sought to characterize the antigen specific immune response to influenza following vaccination with a viral-vectored nucleoprotein + matrix protein 1 (NP+M1) influenza vaccine and subsequent influenza challenge and determine whether there was any change in the phenotype and functional potential of antigen specific CD8+ T cells. We found that following vaccination with modified vaccinia Ankara (MVA)-NP+M1, antigen specific $M1_{58–66}$ CD8+ T cells showed a more enhanced activation profile showing higher levels of perforin, granzyme A and CD57. There was also a reduction in BCL2 expression. These antigen specific cells expanded in response to challenge with live influenza virus. The vaccine-stimulated cells were altered in terms of their surface and intracellular phenotype. Examination of the phenotype of antigen specific T cells may be a useful adjunct for human immunization studies.

MATERIALS AND METHODS

Ethical Permissions and Human Studies. Volunteers were recruited using an approved Medicines and Healthcare products Regulatory Agency and the Oxfordshire Research Ethics committee protocol, and enrolled only after obtaining written informed consent (www.clinicaltrials.gov identifier: NCT00993083, approved 19 May 2009). Volunteers aged 18–45 were recruited at the Centre for Clinical Vaccinology and Tropical Medicine, Oxford and the Welcome Trust Clinical Research Facility, Southampton beginning on 8th June 2009. Volunteers were initially screened by haemagglutination-inhibition assay against the virus to be used in the influenza challenge phase of the study. Those with a titre ≤1:10 were eligible for further screening. Volunteers were seronegative for HIV, Hepatitis B virus and Hepatitis C virus and had not received seasonal influenza vaccination for at least one year prior to enrolment. Routine haematological and biochemical tests on enrolled volunteers were all within normal limits (16).

Vaccine, Vaccinations and Virus Challenge. The CONSORT flow chart for the trial is shown in Figure 1. Beginning 27 July 2009, an MVA vaccine (17) expressing influenza (H3N2 A/Panama/2007/99) NP+M1 was administered, 1.5×10^8 plaque-forming units (PFUs), to 11 human volunteers 1 month before challenge with influenza (H3N2 A/Wisconsin/67/ 2005). Control subjects were challenged with H3N2 virus only. The viral challenge study was conducted by Retroscreen Virology Ltd (16). Symptoms and virus shedding were monitored. Differences between IFN-γ ELISPOT analysis pre and post challenge are given in (16). To examine the acute response after virus infection, blood was taken one day before (21) and days 4 and 7 after challenge, transported to Oxford and then flow cytometry performed on whole blood.

Flow Cytometry Analysis of Whole Blood Samples. Whole blood was aliquoted into tubes and then labelled with six different antibody panels all with tetramer-PE: HLA-A*0201 complexed with $M1_{58–66}$ peptide GILGFVFTL, produced in house using standard methods [18] and incubated for 15 mins at 37uC. Red blood cells were lysed using RBC lysis media (Becton Dickinson, Oxford UK) for 15 mins at room temperature then washed 26 with FACS buffer PBS (Difco, Poole) with 1% v/v BSA (Sigma, Poole UK) followed by incubation with CD8-PerCP and CD4-Pac Blue (eBiosciences, Hatfield UK) plus different panels of ab: CD28-FITC. HLA-DR-APC, CD38-PE-Cy7 and CD27-APCH7 (eBiosciences) or CD57-FITC and CD25-APC or CD45RA-FITC, CD45RO-PECy7 and CCR5-APC. Cells allocated to the Intracellular panels were permeabilised with Perm2 (BD) for 15 mins and washed 26 in FACS buffer. Cells were then labeled with CD8-PerCP (Biolegend) and CD4-Pac Blue followed by: Perforin-FITC (D48, Genprobe, Manchester, UK) or GranzymeA-FITC or GranzymeB-FITC or Ki67-FITC. Cells were then washed twice and fixed in BD cellfix. All abs were from Becton Dickinson (Oxford, UK) unless otherwise stated. Similar staining protocols were also done using the CMV lower matrix protein pp65495–503 NLVPMVATV tetramer (19). Cell events were collected on a 9 colour Cyan Cytometer (Dako, Ely, UK) and data files analysed using FlowJo (Tree Star Inc, Ashland, OR, USA). Data were analysed using a forward side scatter gate followed by CD8 gating then tetramer gating within the CD8+ population. These cells were then analysed for percentage expression of a particular marker using unstained and CD8+ tet- populations to determine where to place the gates. Single colour samples were run for compensation and fluorescence minus one (FMO) samples were also run to check positive and negative populations as well as channel spillover. To monitor overall changes of white blood cell numbers, whole blood samples

were analysed using the BD Trucount system measuring CD4, CD8 and CD3 positive lymphocytes according to manufacturers instructions.

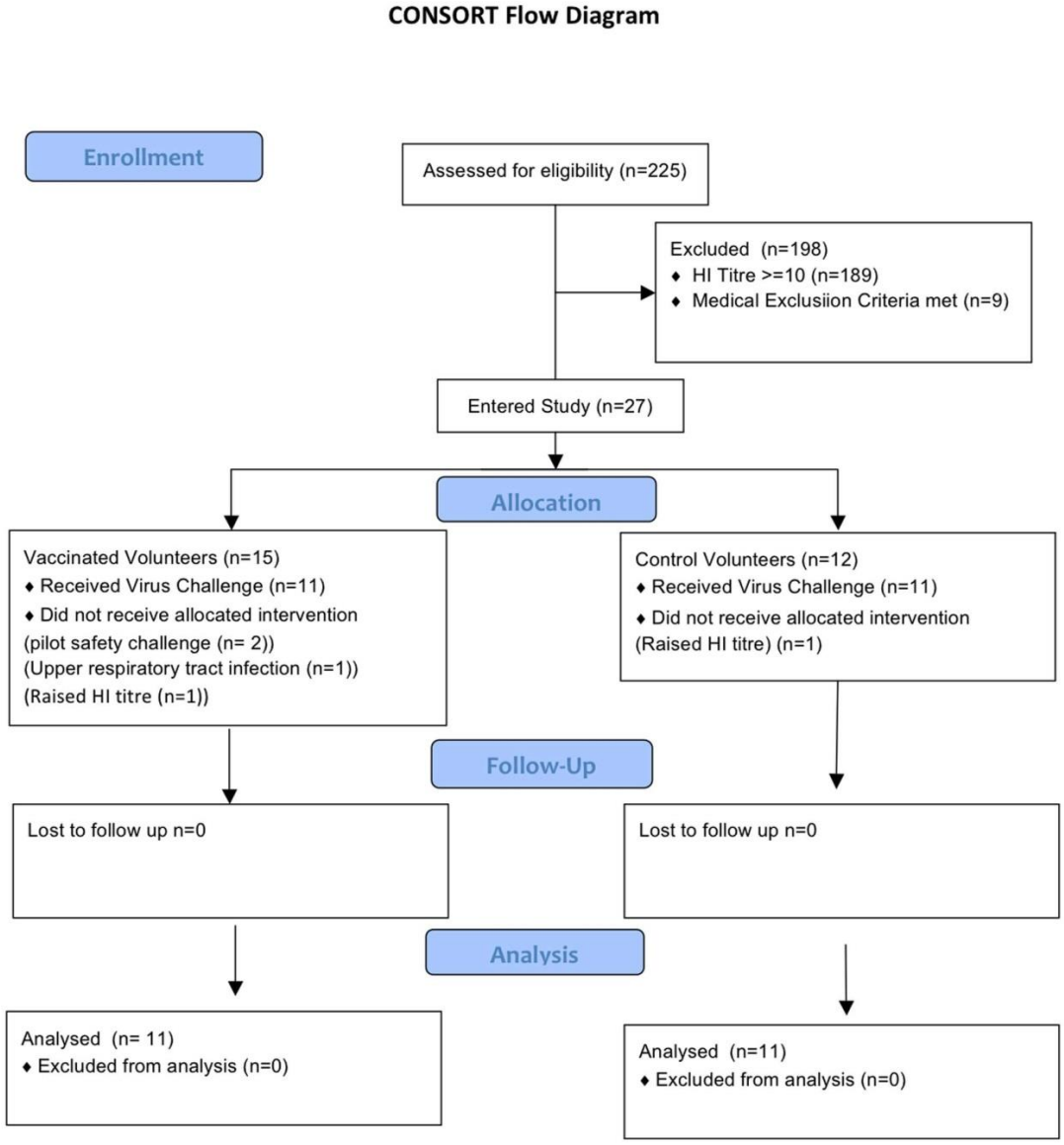

Figure 1. CONSORT flow diagram of the clinical trial.

ELISPOT Analysis of PBMC. PBMC were separated on density gradients and incubated with peptide pools from each gene of the H3N2 virus along with peptide pools from other subtypes of haemagglutinin (HA) and neuraminidase (NA) as described previously (16). Peptide pools were similar to those described previously (20). Swine origin (SO) HA and NA were overlapping 18–20 mers from the full sequence of A/Cal/04/2009 H1N1 influenza virus.

Statistical Analyses. Groups of data were analysed by repeated measures ANOVA using pairing of samples using the statistical package R (R Foundation). Changes between vaccinated and control were considered along with time and interaction between both. Any analyses showing p values <0.05 were considered significant. Data from repeated measures ANOVA are shown in Table S1.

RESULTS AND DISCUSSION

Initially we analysed the percentage and absolute numbers of tetramer positive CD8 T cells for each HLA-A*02 volunteer by FACS on the day before and up to day 7 post challenge. Surprisingly we found that the percentage of $M1_{58–66}$ specific cells, shown in Figure 2A, was not different between vaccinated and control donors. A representative flow cytometry profile from one vaccinated and one control donor is shown in Figure 2B, which shows cells gated on CD8 and the percentage of tetramer positive cells within that gate. The absolute number of $M1_{58–66}$ antigen specific $CD8^+$ T cells was also calculated using CD8 counts from the Trucount and these were not significantly different between groups (data not shown). Overall T cell responses to overlapping peptides spanning the entire H3N2 proteome (5) were tested and no significant differences were observed between vaccinated and control groups to most proteins, despite raised responses to NP in the vaccinated group before infection (Figure S1) (16,17). There is an overall trend of elevation of T cell responses in both groups 7 days after the challenge, which could mainly be CD4 dependent responses as described by Wilkinson et al., (21).

Since the number or proportion of antigen specific cells was not different between the groups we then examined the cell surface and intracellular phenotype of the $M1_{58–66}$ $CD8^+$ T cells. We examined the expression of CD27, CD28, CD38 and HLA-DR on the surface of the cells that are markers associated with activation and differentiation

(19). We found that the expression of CD27, CD28, CD38 and HLA-DR were not different between vaccinated and control donors by repeated measures ANOVA (Figure 3A). The levels of CD57, which is a marker associated with either senescence or activation were different by repeated measures ANOVA (p = **0.00705) and CD57 was enhanced on cells from vaccinated volunteers. Double $CD27^+$ $CD28^+$ positive cells were not different between the groups and a representative flow cytometry profile is shown in Figure 3B.

Further to the cell surface molecules we examined the intracellular molecules, granzyme A, granzyme B and perforin on $M1_{58–66}$ specific cells; which are all associated with better levels of cytolytic activity. Significant changes in perforin expression were only detected using the more sensitive mab D48 that detects newly synthesized perforin (22) and we found that the perforin levels were higher in vaccinated volunteers by repeated measures ANOVA (p = **0.0027) (Figure 3C). We found that granzyme A was elevated in vaccinated volunteers (repeated measures ANOVA p = **0.0025). Granzyme B was not different (Figure 3C). These data imply that the vaccinated donors possess antigen specific cells that have developed the potential to be more cytolytic and this would then potentially correlate with faster virus clearance. In donors vaccinated followed by influenza challenge, levels of B Cell lymphoma-2 (BCL_2) protein were reduced (repeated measures ANOVA p = ***0.00314) as shown in Figure 3C. These changes may indicate more differentiated cells or cells that are more likely to apoptose. Figure 3D shows a flow cytometry profile from one vaccinated and one control donor showing increased perforin (D48 clone) in tetramer positive cells from a vaccinated donor at day four.

Shown in Figure 4A is the phenotype data that we obtained from the vaccinated/control volunteers and then influenza infected volunteers examining different surface and intracellular markers. Overall there are a number of proteins that show trends of difference that are similar to the statistically different changes shown in earlier figures, illustrating an overall picture that the $M1_{58–66}$ specific $CD8^+$ T cells are more responsive from the vaccinated volunteers than those from the control volunteers. Analysis of total $CD4^+$ or $CD8^+$ T cell populations could be useful but because of the unknown specificity of these cells we considered that it was better to measure either influenza or CMV antigen specific cells.

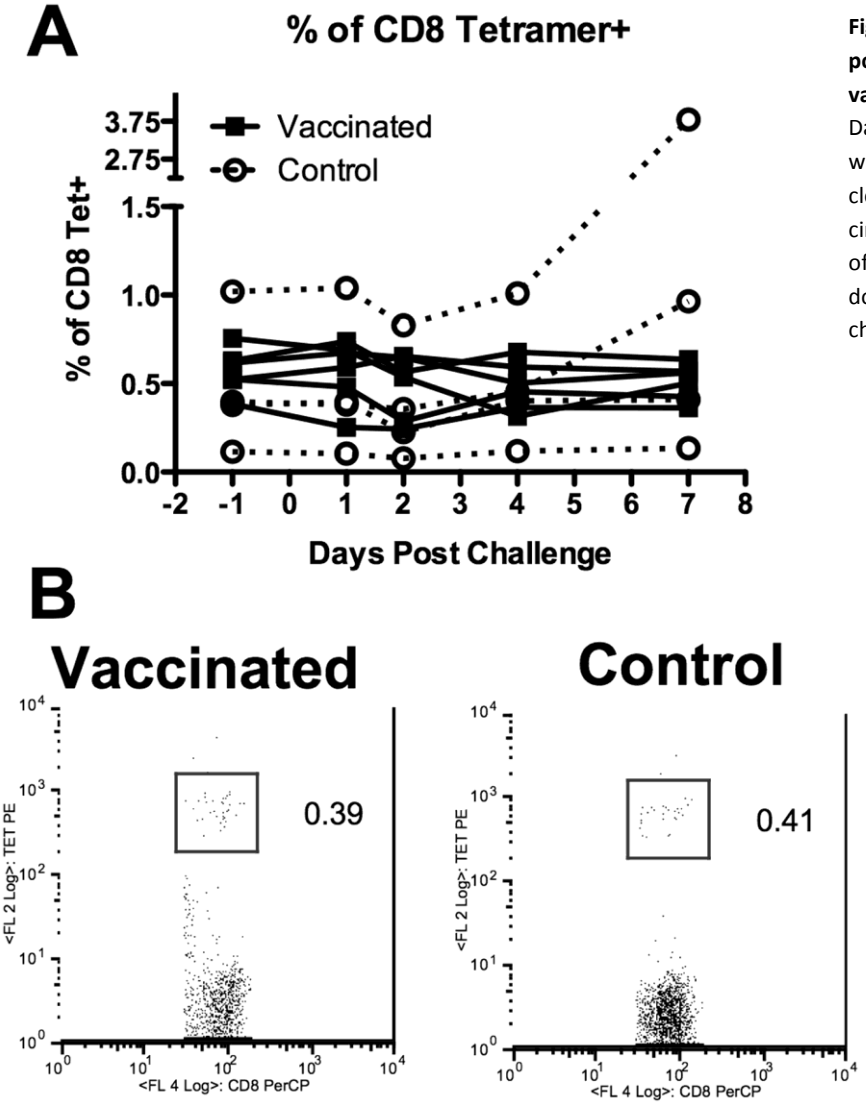

Figure 2. Frequency of tetramer positive cells is similar between vaccinated and control volunteers. Data shows percentage of CD8 cells within a CD8 gate with vaccinated as closed squares and control open circles. B) Representative FACS profile of one vaccinated and one control donor at day 21, one day before challenge with influenza virus.

We also examined the phenotype of CMV specific cells in two vaccinated volunteers that were positive for the CMV tetramer and found that these did not change during the influenza infection (Figure 4B). This indicated that there was no bystander activation of CMV specific T cells during the challenge and that it is unlikely that the adjuvant effects of MVA are causing these changes in phenotype of $M1_{58–66}$ specific cells.

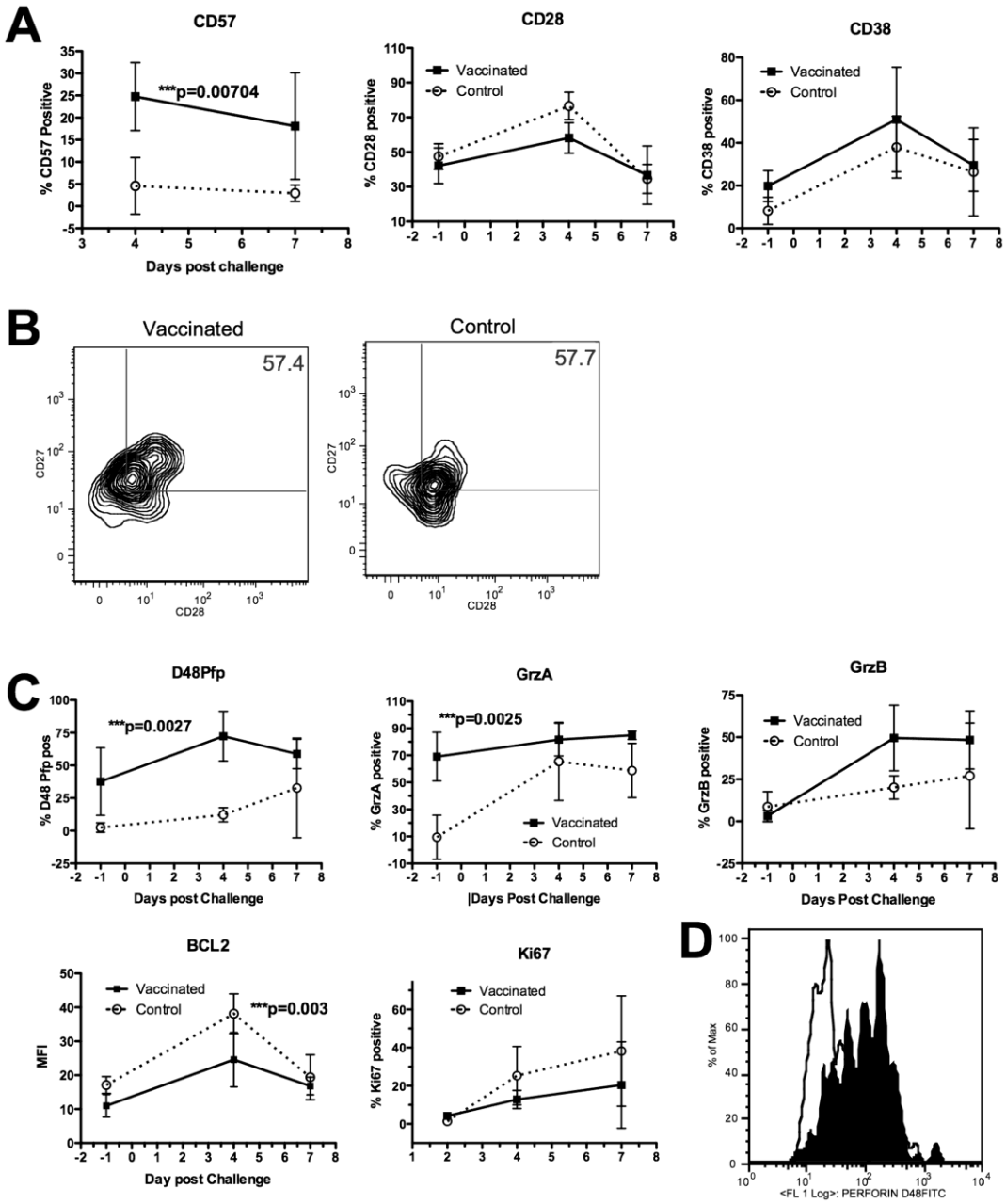

Figure 3. Surface and intracellular activation markers are enhanced on tetramer labeled cells from vaccinated donors compared to control. A) Time course between day 21 and 7 and expression of noted markers on $M1_{58–66}$ tetramer labeled cells. B) Representative flow cytometry plot of $M1_{58–66}$ tetramer positive cells labeled for CD27 and CD28 on day 21 showing similar profiles. C) Graphs plot the percentage of tetramer+ cells or MFI of tetramer+ cells with the noted markers. D) Representative flow cytometry plot of two donors showing control (open plot) and vaccinated volunteer (filled histogram) labeled with anti-D48 Pfp on day 4. Groups were compared using repeated measures ANOVA.

This phase IIa study of a novel influenza vaccine was designed to test whether stimulating NP+M1 T cell responses were able to protect vaccinees against influenza (16), but here we particularly concentrate on changes in surface phenotype of the antigen specific cells after vaccination and influenza challenge. The phenotype of the influenza specific T cells was altered and these changes or enhancements of T cell phenotype have been found in other studies to be associated with protection against influenza (11). Miller et al., found that antigen specific T cells were stimulated in a study of yellow fever and vaccinia vaccination and that vaccinated donors had enhanced activation profiles (23). In other studies enhancement of perforin expression has been demonstrated on IFN-c secreting cells after influenza vaccination (24). We also find that perforin level is increased on antigen specific cells identified using tetramers after vaccination, which gives more insight into the response of antigen specific T cells after vaccination and challenge in humans.

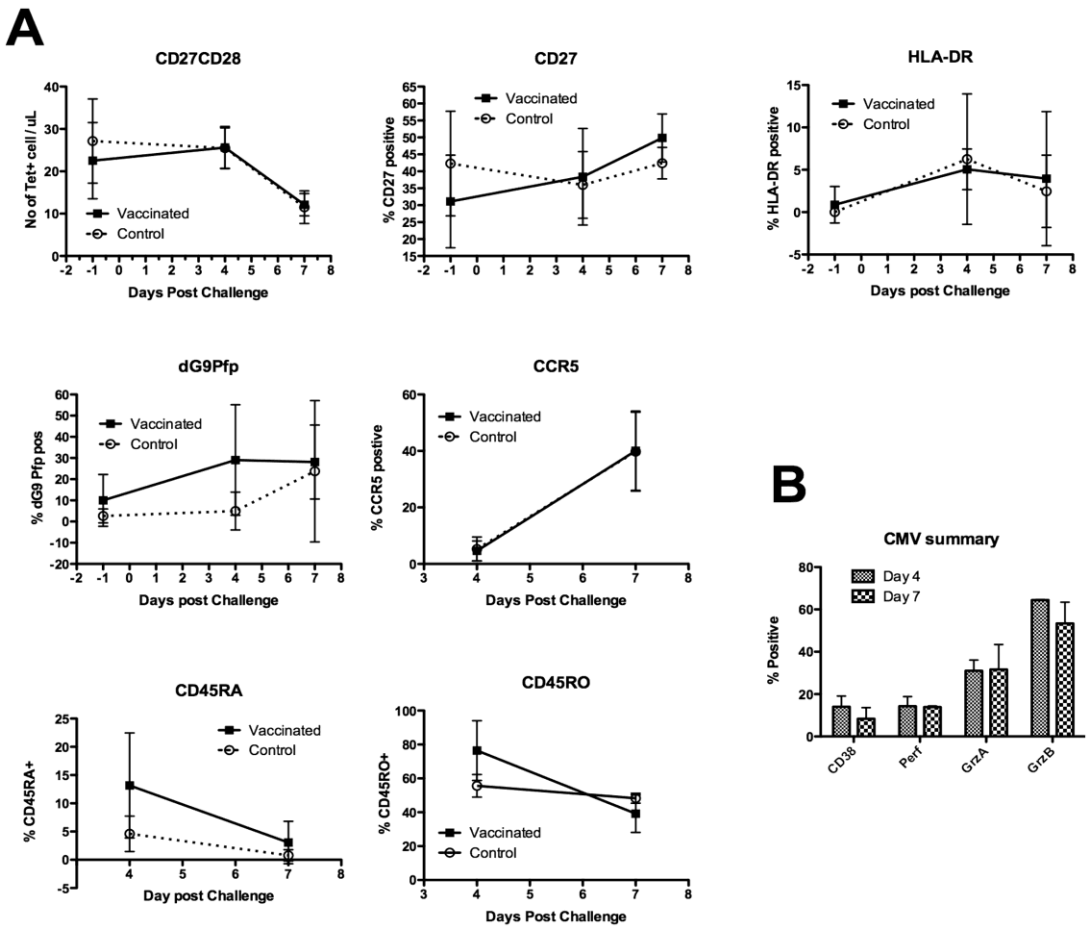

Figure 4. Analysis of markers that are not different between vaccinated and control volunteers and analysis of CMV specific T cells after influenza challenge. A) Cells were labeled for flow cytometry and percentage positive calculated using FlowJo. All donors are shown in the figures and indicate positivity for various markers after vaccination and or challenge with influenza virus. All groups were compared using repeated measures ANOVA. B) Analysis of phenotypic markers on CMV tetramer positive CD8 T cells showing different marker expression on these antigen specific cells.

The M1 specific $CD8^+$ T cells have been shown to be protective in A2 transgenic mice (8), and HLA A2 positive donors commonly have detectable $M1_{58–66}$ specific CD8 T cells (9). Terajima et al., and Tu et al., (25,26) show the presence of influenza specific T cells in human samples that have the potential to protect against novel strains of influenza. McMichael has shown association between cytotoxic activity of T cells and reduced virus shedding in humans (7). Hikono et al., have shown that certain activated memory T cells can protect against subsequent influenza challenge (11) and that activation phenotypes may be more important than absolute numbers of memory cells. Murine antigen specific $CD8^+$ T cells of a single specificity can be protective against influenza challenge after adoptive transfer (10) or by priming with a known peptide (8). In humans, protection against influenza is likely to involve different specificities of $CD4^+$ and $CD8^+$ T cells since it is likely that most adults will have previously been exposed to influenza. These studies could be extended in future to examine different specificities and HLA alleles. Another observation is that there are differences in T cell phenotype when the ELISPOT responses are similar. Despite the usefulness of the ELISPOT assay and its widespread use in trials of this type, it only gives one view of the T cell response and we recommend a more detailed T cell phenotype analysis to provide a more complete description of the effects of vaccination.

In conclusion, we have found that vaccination with an MVA construct containing NP+M1 in healthy volunteers led to more activated antigen specific $CD8^+$ T cells and these cells have the potential to be more active in clearing virus because of higher levels of perforin and granzyme proteins. The approach of using tetramers in combination with phenotypic markers may also be a useful method to assess the immunogenicity of different vaccines.

SUPPORTING INFORMATION - Figure S1 ELISPOT responses using peptides from individual genes indicate rise in NP response after vaccination & infection and rise of H3 HA response after challenge. IFN-γ ELISPOT assays were done using standard methods using overlapping peptides from H3N2 and other strains of influenza viruses.

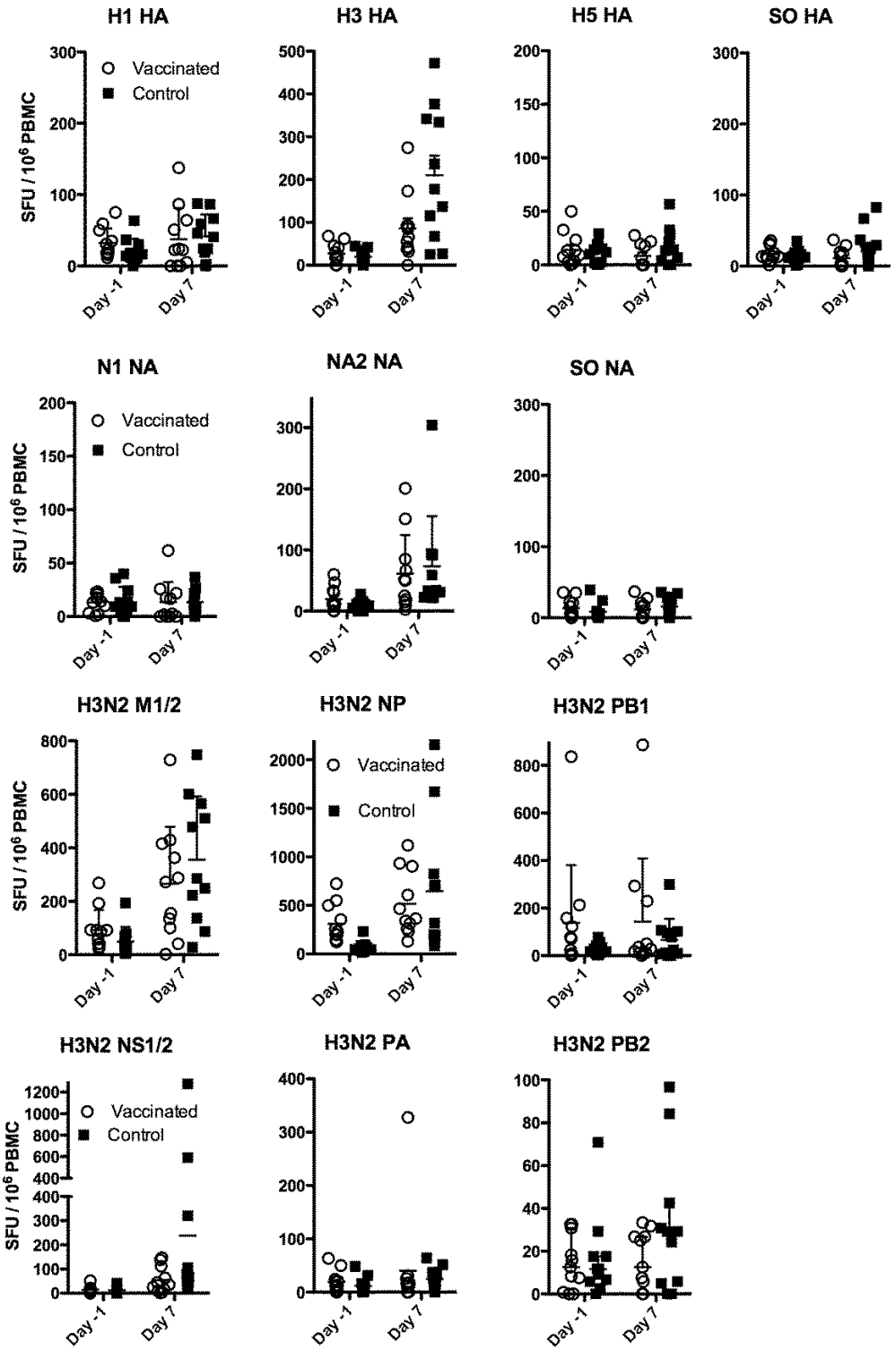

Table S1 Summary of repeated measures ANOVA p values of marker analysis on $M1_{58–66}$ specific CD8 T cells after vaccination and/or challenge

Marker[a]	Vaccine[b]	Time[c]	Interaction[d]
CD57	0.00704	0.085	0.333
CD28	0.166	1.33e-05	0.0947
CD38	0.252	0.00015	0.632
Pfp D48	0.00275	0.00870	0.126
GrzA	0.00250	0.000201	0.00879
GrzB	0.0971	5.13e-05	0.0343
BCL2	0.00313	1.34e-05	0.113
Ki67	0.230	0.0039	0.265
CD27/28	0.618	0.0001	0.592
CD27	0.942	0.0249	0.0868
HLA-DR	0.833	0.106	0.824
Pfp dG9	0.254	0.0143	0.233
CCR5	0.970	5.88e-5	0.918
CD45RA	0.0943	0.0122	0.228
CD45RO	0.389	0.00176	0.0288

[a]Analysis of markers examined on antigen specific T cells after vaccination with an NP+M1 vaccine or control donors followed by challenge with intranasal influenza vaccine.

[b]p value with respect to variation caused by vaccine.

[c]P values with respect to variation attributable to time.

[d]p of both time and vaccine together.

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8.5 Paper 5 - Severity Assessment of Influenza Virus Infection in Secondary Care

Patrick J Lillie, Rohit Bazaz, Laura Dexter, Charles Biju, Lucy Peart, Leon Lewis, Mohammad Raza, Paul Whiting, Anne Tunbridge

We read the comprehensive review on pH1N1 severity assessment by Singanayagam and colleagues with interest (1). They highlight the limitations of severity assessment tools and factors associated with severe disease with pH1N1 infection, as had previously been shown for influenza prior to pH1N1 (2). The majority of evidence recently has concentrated on pH1N1 infection only, most of which was collected during the first wave of cases in 2009/2010 in both the UK (3) and elsewhere (4-8). It is not clear how applicable this is in the setting of pH1N1 becoming the predominate seasonal influenza virus, particularly with co-circulation of influenza B virus, as was the case in the UK in the winter of 2010-2011 (9). Here we describe factors associated with admission to critical care in adult patients attending our hospitals with confirmed influenza, of any type, during the winter of 2010-2011. We also propose an admission and severity assessment pro forma which we intend to use prospectively in the forthcoming influenza season.

Cases of influenza infection in adults were retrospectively identified from the virology laboratory records; demographic details and requirement for critical care were available for all adult patients admitted into our hospital trust. More in-depth assessment was made of a smaller cohort from retrieved notes. Data were analysed using Graphpad Prism and SPSS version 19.

Over the winter season of 2010-2011 the virology department of Sheffield Teaching Hospitals Foundation NHS Trust identified 418 separate samples as being PCR positive for influenza virus from the 1st November 2010 to the 31st January 2011. Cases peaked over the Christmas period with 217 confirmed influenza infections in the 2 weeks from the 20th December. Of the total isolates, 312 were influenza A (all typed as pH1N1) and 106 were influenza B. Demographic data and requirement for critical care were available on all these cases. There was no significant difference in the median age between the influenza A and B groups (47 years and 50 years respectively $p = 0.198$) or in the need for critical care (28/312 influenza A and 4/ 106 influenza B, $p = 0.093$).

Case notes for 131 patients were retrieved for further evaluation. The most common presenting features were current fever or history of fever (87.6%) and cough (92.4%). Gastrointestinal symptoms were common, with 28.2% of cases presenting with vomiting and 19.8% with diarrhoea. 38 patients had no risk factors for severe influenza infection. Of the 131 patients, 27 patients required critical care, of whom 9 died. Table 1 shows the variables associated with critical care admission on uni and multi-variate analysis.

	Critical care case (n=27)	Non critical care case (n=104)	P value - univariate	P value – multivariate
Age	49 (37-65)	34.5 (25-53)	0.0033	>0.05
Oxygen saturations >92% on air	4 (16%)	84 (80.8%)	<0.0001	<0.0001
Respiratory rate	28 (22-38)	20 (17-24)	<0.0001	0.035
Existing respiratory illness	14 (51.9%)	26 (25%)	0.0099	>0.05
Smoker	14 (51.9%)	26 (35.6%)	0.0185	>0.05
Obesity	6 (22.2%)	6 (5.8%)	0.0171	>0.05
Pulse	112 (98.5-131.5)	100 (88-114)	0.006	0.036
Confusion	12 (44.4%)	7 (6.7%)	<0.0001	>0.05
CXR abnormal	24 (92.3%)	24 (24.7%)	<0.0001	>0.05
Urea (mmol/L)	8.6 (3.6-17.1)	3.5 (2.4-4.6)	<0.0001	>0.05
Albumin (g/L)	30 (26-33)	37 (34-41)	<0.0001	0.001
CRP (mg/L)	167.3 (59-247.5)	51.3 (22.7-120.3)	0.0123	>0.05

Table 1: Features predicting need for critical care. Values are medians (interquartile range), or absolute numbers (%). CXR – chest x-ray, CRP – C Reactive Protein. Fishers exact test or Mann-Whtiney U test as applicable.

A total of 802 bed days were used, with a median length of stay of 3 days (range 1 - 70).

Empiric treatment with neuraminidase inhibitors, as compared to no treatment or treatment initiated after receipt of a positive PCR result, was associated with a reduction in median length of stay from 3 to two days ($p = 0.0107$).

Thirty-two of the 131 patients were discharged within 24 hours, and potentially may have been suitable for management in the community with antivirals. Compared to those admitted for over 24 hours none of these 32 patients were aged over 65 (median age 28.5 years in 24 h group, 45 years in>24 h group, p = 0.0033), none were confused (19/99 in the >24 h group, p = 0.0038), all had oxygen saturations on room air of >92% (40/97 in the >24 h group, p < 0.0001) and they had lower respiratory rates (median respiratory rate 18 in the 24 h group, 20 in the >24 h group p = 0.0014). Using the data from both the critical care and short stay groups, we have drafted an assessment pro forma for use in the coming influenza season, to assist our admission units in deciding on appropriate disposal of patients. The pro forma when applied retrospectively to this cohort of 131 patients had good predictive ability for need for admission, with very good predictive ability of the need for critical care (Figure 1).

As the northern hemisphere prepares for the next influenza season, variations in the type of influenza circulating and the proportion of people susceptible differ between areas. Use of assessment tools such as ours may improve use of critical care services, which can come under great pressure in times of influenza activity (5). Conversely, early identification of patients who could be managed in the community, with appropriate antiviral treatment (10) may release resources in secondary care to manage more severe cases.

In summary, simple clinical variables and investigations allow patients with influenza infection to be assessed rapidly and appropriately, and empiric treatment may shorten duration of admission.

Does the patient need to be admitted?

- Confused? Y / N
- O_2Saturations<93%? Y / N
- Aged ≥65? Y / N
- Respiratory rate >24 Y / N
- Any co-morbidity? Y / N

- If no to all the above, then may be suitable for management in the community with antiviral medication.
- If yes, then refer for assessment including CXR and blood tests

For predicting admission of ≤24 hours, Sensitivity 53.1%, Specificity 86.9%, Positive predictive value 56.7%, Negative predictive value 85.2%

Does the patient require critical care?

- Respiratory rate >30 Y / N
- Abnormal Chest x-ray Y / N
- O_2 Saturations<90% Y / N
- Albumin <33g/l Y / N
- Urea >8mmol/l Y / N
- CRP >150 mg/l Y / N

- If Yes to 2 consider critical care review.
- If yes to 3 or more, critical care review strongly advised.

For predicting need for critical care admission , using 2 or more as a cut off, Sensitivity 88.9%, Specificity 81.7%, Positive predictive value 55.8%, Negative predictive value 96.6%

Figure 1 Assessment pro forma with performance characteristics.

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8.6 Paper 6 - Distinguishing Malaria and Influenza: Early Clinical Features in Controlled Human Experimental Infection Studies

Patrick J. Lillie[1], Christopher J.A. Duncan[1], Susanne H. Sheehy, Joel Meyer, Geraldine A. O'Hara, Sarah C. Gilbert, Adrian V.S. Hill

1 – Joint first authors

SUMMARY

During the H1N1 influenza pandemic (pH1N1/09) diagnostic algorithms were developed to guide antiviral provision. However febrile illnesses are notoriously difficult to distinguish clinically. Recent evidence highlights the importance of incorporating travel history into diagnostic algorithms to prevent the catastrophic misdiagnosis of life-threatening infections such as malaria.

We applied retrospectively the UK pH1N1/09 case definition to a unique cohort of healthy adult volunteers exposed to *Plasmodium falciparum* malaria or influenza to assess the predictive value of this case definition, and to explore the distinguishing clinical features of early phase infection with these pathogens under experimental conditions.

For influenza exposure the positive predictive value of the pH1N1/09 case definition was only 0.38 (95% CI: 0.06-0.60), with a negative predictive value of 0.27 (95% CI: 0.02-0.51). Interestingly, 8/11 symptomatic malaria-infected adults would have been inappropriately classified with influenza by the pH1N1/09 case definition, while 5/8 symptomatic influenza-exposed volunteers would have been classified without influenza ($P = 0.18$ Fisher's exact). Cough ($P = 0.005$) and nasal symptoms ($P = 0.001$) were the only clinical features that distinguished influenza-exposed from malaria-exposed volunteers. An open mind regarding the clinical cause of undifferentiated febrile illness, particularly in the absence of upper respiratory tract symptoms, remains important even during influenza pandemic settings. These data support incorporating travel history into pandemic algorithms.

INTRODUCTION

With the advent of the first pandemic of influenza of the 21st century, there was significant concern about the potential impact on healthcare infrastructure of managing pandemic H1N1 (pH1N1) infections in traditional settings. In July 2009, due to sustained community transmission of pH1N1 (with 80,000-100,000 symptomatic cases/week in England (1), control efforts shifted to mass treatment strategies, and the UK National Pandemic Flu Service began to offer treatment of symptomatic individuals identified by telephone or internet-based triage in England (2). However the initial pandemic influenza triage algorithm omitted questions about travel to a malaria endemic area (3). As a result, at least 3 cases of malaria infection in travellers that were misdiagnosed with influenza were reported (4,5). While in a pandemic setting such triage may be essential for efficient recognition and management of cases, the UK case definition has been subsequently shown to be poorly predictive (2).

In the course of separate candidate vaccine efficacy trials carried out by our unit (www.clinicaltrials.gov NCT00890760/NCT00993083), we independently exposed cohorts of unimmunised healthy control volunteers to pathogenic strains of either *Plasmodium falciparum* (*P. falciparum*) malaria or influenza A under controlled experimental conditions, and followed them closely in the early phases of infection until clinical diagnosis. These control volunteers were enrolled to ensure the reliability of the respective experimental infections and therefore did not receive any immunisations. Although these studies were conducted separately, assessment of clinical symptoms was conducted according to uniform criteria. These data afford us a unique opportunity to perform a retrospective comparison of the early clinical features that may be of use in differentiating clinically between an often uncomplicated illness in influenza and a potentially life-threatening infection in malaria, and to assess whether the clinical features included in the UK pandemic case definition alone were sufficiently discriminatory.

METHODS

Controlled human malaria infection (CHMI). Recruitment occurred at the Centre for Clinical Vaccinology and Tropical Medicine, Oxford, with the challenge procedure performed, using five infectious bites from *P. falciparum* 3D7-strain infected mosquitoes, at Imperial College, London. We recruited healthy malaria naïve adults aged 18-50 years old from the Oxford area. Enrolled control volunteers ($n = 12$) were seronegative for HIV, Hepatitis B virus and Hepatitis C virus and were used to assess the infectibility of the challenge inoculum. Routine haematological and biochemical tests on enrolled control volunteers were all within normal limits. These control volunteers underwent full clinical examination and safety blood tests the day prior to sporozoite challenge. All volunteers remained outpatients throughout the challenge procedure. Volunteers attended twice daily for clinical assessment as previously described (6). Self-reported solicited and unsolicited symptoms and routine observations (BP, pulse, temperature) were collected twice daily from day 6.5 post-challenge and guided physical examination carried out as indicated by symptoms. Thick blood films were examined twice daily for malaria parasites by blinded microscopists together with a concurrent highly sensitive quantitative polymerase chain reaction (qPCR) assay for *P. falciparum*. Treatment with a standard oral dose of artemether/lumefantrine for 3 days was administered on the detection of a single parasite by microscopy, or on the occurrence of significant symptoms + sequential positive qPCR (if blood films were negative). Volunteers were followed up daily until two consecutive negative malaria films were observed, and returned for full clinical assessment including safety blood tests on days 35, 90 and 140 post-challenge.

Controlled human influenza infection. Recruitment occurred at the Centre for Clinical Vaccinology and Tropical Medicine, Oxford and the Welcome Trust Clinical Research Facility, Southampton. Volunteers aged 18-45 years were initially screened by haemagglutination inhibition (HI) assay against the virus to be used in the challenge phase of the study to ensure susceptibility to challenge. Those with a titre ≤1:10 were eligible for further detailed screening. Enrolled volunteers ($n = 12$) were seronegative for HIV, Hepatitis B virus and Hepatitis C virus and had not received seasonal influenza vaccination for at least one year prior to enrolment. Routine haematological and biochemical tests on enrolled control volunteers were all within normal limits. Control volunteers underwent clinical examination, spirometry, safety blood tests and

electrocardiography on entry to the quarantine facility. Two days after entry to quarantine (to allow time for observation of any symptoms of respiratory virus infections) control volunteers were challenged with intra-nasal administration of H3N2 influenza (A/Wisconsin/67/ 2005) at a dose of 1 ml of $10^{5.25}$ TCID50/ml. All volunteers were inoculated during the same 2- h period. After challenge, volunteers were followed up in the quarantine facility as previously described (7). Self-reported symptoms were collected twice daily and a physical examination by a blinded physician was carried out daily. Nasal lavage fluid for quantification of viral shedding was obtained daily. Symptoms and physician elicited clinical signs were recorded using a standardised modified Jackson scoring system (8) (which assigns severity of symptoms such as cough, rhinorrhoea etc., on a scale of 0-3, where no symptoms = 0; just noticeable = 1; bothersome but can still do activities = 2; and bothersome and cannot do daily activities = 3) with a score of >4 indicating influenza disease. Safety blood tests and further spirometry and electrocardiography were performed on all volunteers whilst in quarantine. A five-day course of oseltamivir was commenced from day 5 post-challenge for all volunteers with medication provided to complete the course after discharge. Volunteers were released from quarantine on the 7th day post-challenge after a negative rapid antigen test for influenza on nasal washings was obtained. After discharge all volunteers were followed up on days 35, 91 and 181 post-challenge for safety blood tests.

Ethics. Clinical trial protocols (www.clinicaltrials.gov identifiers: NCT00890760 and NCT00993083) were approved by the UK Medicines and Healthcare Products Regulatory Agency and the Oxfordshire NHS Research Ethics Committee. All volunteers provided signed informed consent prior to any study procedure, and all studies were conducted according to the principles of Good Clinical Practice and the Declaration of Helsinki.

Case definition. We examined the UK 2009 pandemic H1N1 influenza A case definition (pH1N1/09) which was used from 2nd July 2009 to determine provision of antiviral therapy in the absence of assessment by health professional.

This was as follows:

- Fever >38 ^{O}C or history of fever,

and two or more of the following:

- Cough, sore throat, headache, rhinorrhoea, limb or joint pain (2,9).

Statistical analysis. We calculated the ability of the UK pH1N1/09 case definition to distinguish influenza-exposed unvaccinated volunteers from malaria-exposed unvaccinated volunteers by calculating positive and negative predictive values. We also analysed the significance of differences between proportions of early symptoms in the group of malaria-exposed and influenza-exposed volunteers, and repeated this analysis for those individuals with laboratory-confirmed influenza. Continuous variables were assessed for normality and significance of differences between central tendencies (mean or median) assessed by appropriate parametric or non-parametric tests (Student's *t* test or Manne Whitney *U* test respectively). All statistical analysis was performed using Prism 5.0 (GraphPad), with two-tailed tests and an alpha value of <0.05 considered statistically significant.

RESULTS

Participants. 1 out of 12 influenza unvaccinated control volunteers developed an asymptomatic rise in HI titre and this volunteer was excluded prior to challenge; therefore 11 unvaccinated controls were exposed to influenza infection. 11/12 malaria unvaccinated control volunteers were included in the analysis. No serious adverse events occurred in volunteers in either challenge. Demographic details of these participants are summarised in Table 1. All malaria volunteers were diagnosed on the basis of positive blood film microscopy (geometric mean parasitaemia 4030p/ml by qPCR) for *P. falciparum* a mean of 11.8 days post-challenge (Ewer K. *et al.*, manuscript submitted). 5/8 symptomatic influenza volunteers developed laboratory-confirmed influenza infection, defined as positive viral culture for challenge virus on nasal lavage fluid a mean of 2.3 days post-challenge (Lillie P.J. *et al.*, Clinical Infectious Disease *in press*).

Demographics	Malaria	Influenza	P value
Number of volunteers	11	8	-
Median age (IQR)	28.6 (22.5-35.4)	28 (23-37)	0.51
Male / Female	6 / 5	3 / 5	0.65

Table 1 - Demographic details of symptomatic controlled experimental infection volunteers.

Clinical features. Clinical features of the exposed volunteers in the influenza and malaria cohorts are summarised in Table 2. Only the incidence of cough (P = 0.005 Fisher's exact) and rhinorrhoea (P = 0.001 Fisher's exact) were significantly different between the groups. In line with this increase in upper respiratory tract symptoms in influenza-exposed volunteers, there was also a trend towards an increased frequency of sore throat (P = 0.06 Fisher's exact). Restricting analysis to the 5/8 symptomatic influenza-exposed volunteers with viral culture-confirmed infection, the significant increase in cough (P = 0.003 Fisher's exact) and rhinorrhoea (P = 0.001 Fisher's exact) remained, whilst the trend for sore throat became significant (P = 0.02 Fisher's exact). Physiological parameters did not distinguish the malaria and influenza cohorts. There were no significant differences in the peak mean heart rate (malaria 92.2 beats per minute [95% CI: 82.6-101.8], influenza 82.7 [95% CI: 69.5-96.0], P = 0.19 t test) or temperature (malaria 37.2 °C [95% CI: 36.6-37.7], influenza 37.3 [95% CI: 36.9-37.7], P = 0.72, t test) between the groups, and there were no volunteers with clinically significant hypotension in either group.

Laboratory features. Laboratory analysis was performed at different time-points post-challenge for influenza and malaria-infected volunteers, prohibiting direct comparisons. However, grade 1 thrombocytopaenia and leucopaenia occurred with greater frequency in malaria-exposed volunteers. The diagnosis of malaria was confirmed by thick film microscopy in 12/12 volunteers, all of whom were also positive for *P. falciparum* by qPCR. The diagnosis of influenza was confirmed by viral culture in 5/8 symptomatic volunteers following challenge. There were no significant abnormalities in ECG, laboratory or spirometry parameters post-challenge in the influenza cohort.

Symptom	Malaria infected ($n = 11$)	Influenza exposed[a] ($n = 8$)	*P* value[b]	Influenza infected[a] ($n = 5$)	*P* value[b]
Cough	0	5	0.005	4	0.003
Diarrhoea	2	0	0.49	0	1.0
Headache	10	6	0.55	4	1.0
Fever	8	2	0.18	2	0.55
Malaise	3	3	1.0	2	1.0
Myalgia/ Arthralgia	10	5	0.72	3	0.21
Nasal Symptoms	1	7	0.001	5	0.001
Nausea/ Vomiting	3	2	1.0	1	1.0
Sore throat	0	3	0.058	3	0.018
Met case definition	8	3	0.18	3	1.0

Table 2 - Clinical features of symptomatic individuals following pathogen exposure. [a] 8/11 influenza-exposed volunteers became symptomatic, 5/8 of whom developed viral culture-confirmed influenza infection.[b] Analysis by two-tailed Fisher's exact test versus malaria-infected volunteers.

DISCUSSION

The pH1N1/09 case definition was poorly predictive of influenza infection in this cohort of unimmunised healthy volunteers challenged with *P. falciparum* malaria or influenza A, in keeping with recent data on its performance in clinical practice (2) as well as similar case definitions in other settings (10). In our studies volunteers were treated as soon as significant symptoms or positive blood film developed, therefore only clinical features in the early phase of illness were addressed, and it remains possible that the power to distinguish between these infections clinically might be improved with a longer duration of illness. In addition the use of a non-pandemic challenge strain of influenza in this study may reduce generalisability of findings to pH1N1, which has higher pathogenicity in

younger individuals (11) presumably related to reduced pre-existing immunity (12); yet all volunteers in our study had no detectable pre-existing immunity to the challenge strain, so in this context they might be considered more representative of a pandemic exposed population. Moreover, the pre-assessment probability of infection has a major impact on the PPV and NPV, so during a pandemic setting (where the prevalence of influenza would be significantly greater than malaria) the case definition would be expected to perform significantly better. However in agreement with our data several instances of misclassification of *P. falciparum* (4,5) and other serious illnesses (9) have been reported in the context of protocol-based case-definitions during the peak of community transmission during the pandemic, highlighting the limitations of such protocol or algorithm-based diagnosis (5). Telephone consultation is an important component of infectious disease practice (13), and telephone or internet-based triage has considerable practical advantages in pandemic settings, however the poor predictive capacity of the pandemic case- definition demonstrated here supports the revisions to the case definition to incorporate travel history (3).

Only upper respiratory tract symptoms reliably distinguished malaria from influenza in our study. Whilst the nasal route of inoculation may have theoretically influenced the incidence of rhinorrhoea by delivering a higher multiplicity infection to the nasal mucosa, a recent influenza clinical case series identified cough as a presenting symptom in 92% of individuals with naturally-acquired proven influenza infection (14), suggesting that in the absence of upper respiratory symptoms, particularly cough (15), a presumptive diagnosis of influenza should be reconsidered.

Phase IIa controlled experimental infection studies play an important role in improving our understanding of the clinical features of infectious diseases (16,17). However for ethical reasons sample sizes for such studies are relatively small, thus our statistical power to assess differences in clinical features between malaria and influenza in the analysis presented here may have been reduced. In addition, influenza challenge protocols rarely result in 100% attack rates, (7) as reflected in the 8/11 volunteers developing symptoms and the 5/8 symptomatic volunteers with laboratory-confirmed influenza following nasal challenge, which further impacted sample size. Nevertheless, several lines of evidence support the conclusion based on these data that clinical features alone poorly distinguish between these infections. Firstly there are no reliable clinical diagnostic criteria for influenza (15). Secondly both infections are frequently confused in febrile returning travellers, where influenza is a frequent diagnosis

in travellers to malaria-endemic regions (18-21), as well as in individuals in malaria-endemic regions where a significant burden of undiagnosed influenza appears to exist in holoendemic transmission settings (22). Therefore keeping an open mind regarding the clinical cause of undifferentiated febrile illness, particularly in the absence of upper respiratory tract symptoms, remains important even during influenza pandemic settings.

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Dear Dr Spencer

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Regards

Patrick Lillie

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name…Alexandra Spencer……..

Signed…  ….

Date……14/10/2013…….

Dear Prof Hill

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name Adrian V. S. Hill

Signed… [redacted]

Date…… 6 November 2013

Dear Dr McLaren

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name**…James McLaren**……..

Signed… …….

Date…**14.10.2013**…….

Dear Dr Blais

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name…Marie-Eve Blais……..

Signed..[redacted]….

Date……December 10th 2013…….

Dear Dr Hamill

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name…Matthew Hamill……..

Signed… ……

Date……15th Oct 2013…….

Dear Prof McMichael

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name……Professor Andrew McMichael

Signed……

Date……December 10 2013

Dear Dr Lambkin-Williams

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name…Rob Lambkin-Williams……..

Signed

Date……1/11/2013…….

Dear Dr Lambe

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name…Teresa Lambe……..

Signed…… [redacted] ……..

Date……14.10.13…….

Dear Dr Tunbridge

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Regards

Patrick Lillie

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Severity assessment of influenza virus infection in secondary care. *J Infect* 2012; **64:** 239-241

I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name..Dr AJ TUNBRIDGE

Sign [redacted]

Date.....10/10/13

Dear Dr Milicic

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Name.....Anita Milicic.....

Signed.....[redacted]

Date.....14 October 2013.....

Dear Dr Bazaz

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Name..... ROHIT BAZAZ

Signed.....

Date..... 16/10/2013

Dear Dr Charles

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Name..... Brian Charles

Signed. [redacted]

Date..... 18/11/13

Dear Dr Duncan

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Name..... CHRIS DUNCAN

Signed.....[redacted]

Date..... 24/10/13

Dear Dr Dong

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Name.....Tao Dong .....

Signed[redacted].....

Date.....12-10-2013.....

Dear Dr O'Hara

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Regards

Patrick Lillie

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name O'HARA.....

Signed.... [redacted].....

Date 19/11/13.....

Dear Dr Poyntz

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Name. Hazel Poyntz

Signed. [redacted]

Date. 29/10/2013

Dear Dr Fletcher

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name.....Helen Fletcher

Signed 

Date.....1st November 2013

Dear Dr Meyer

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

Regards

Patrick Lillie

List of co-authored papers

Distinguishing malaria and influenza: early clinical features in controlled human experimental infection studies.*Travel Med Infect Dis* 2012; **10:** 192-196

I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name..... JOEL MEYER.....

Signed.....[redacted].....

Date..... 13/10/13.....

Dear Dr Collins

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Regards

Patrick Lillie

List of co-authored papers

Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. *Clin Infect Dis* 2011; **52:** 1-7

I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name..... KATHARINE COLLINS

Signed.... [redacted]

Date..... 29/10/2013

Dear Dr Ladell

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

Regards

Patrick Lillie

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A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. *PLoS One* 2012; **7:** e48322

I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name KRISTIN LADELL

Signed 

Date 14th October 2013

Dear Dr Dexter

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

Regards

Patrick Lillie

List of co-authored papers

Severity assessment of influenza virus infection in secondary care. *J Infect* 2012; **64:** 239-241

I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name... LAURA DEXTER

Signed... [redacted]

Date... 14/10/13

Dear Prof Gilbert

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinicall features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name.....Sarah C Gilbert.....

Signed..... 

Date.....10.10.2013.....

Dear Dr Gilbert

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name..... Dr Anthony Gilbert

Signed..... [redacted]

Date..... 14 OCT 2013

Dear Dr Ewer

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name... Dr Katie Ewer

Signed.....[redacted]

Date... 10/10/2013

Dear Prof Price

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name DAVID A PRICE

Signed

Date 14 OCT 2013

Dear Dr Peart

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name LUCY PEART

Signed [redacted]

Date 10/10/13

Dear Dr Mullarkey

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name..Caitlin Mullarkey.....

Signed..[redacted]

Date.....4/11/13.....

Dear Dr Whiting

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name.....

Signed...

Date.....15/11/2013.....

Dear Prof Oxford

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name..... PROF J OXFORD

Signed.....

Date..... 7th Nov 2013

Dear Dr Antrobus

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name.....Richard Antrobus

Signed..... 

Date.....21/10/13

Dear Prof Rowland-Jones

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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I agree to the inclusion of the above listed published research articles in Dr Patrick Lillie's PhD by publication thesis, to be submitted to the University of Sheffield.

Name ANN ROWLAND JONES

Signed [redacted]

Date 18/10/13

Dear Dr Faust

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name...Saul N Faust

Signed...[redacted]

Date.....11th Oct 2013

Dear Dr Sheehy

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name..SUSANNE SHEEHY

Signed...[redacted]

Date..11/10/13

Dear Dr Berthoud

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Name..Dr. Berthoud.....

Signed [redacted]

Date.....3.11.2013.....

Dear Dr Havelock

I am currently in the process of applying for a PhD by publication at the University of Sheffield (on influenza virus vaccines and clinical features). As a co-author on the paper(s) listed below I would be very grateful if you would allow me to include the below listed papers in this submission. If you agree to this, could you please sign the bottom of this document and either return to me by email (patricklillie@doctors.org.uk) or by post to; Dr Patrick Lillie, Department of Acute Medicine, Kings Mill Hospital, Mansfield Road, Sutton in Ashfield, Nottinghamshire, NG17 4JL. Thank you for your response, please contact me if there are any questions.

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Name Tom Havelock

Signed [redacted]

Date 14/10/2013.

Dear Dr Peng

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Name.....Yanchun Peng.....

Signed [redacted]

Date.....12.Oct.2013.....

Dear Dr Powell

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Name.....Timothy Powell....

Signed.....[redacted]

Date.....14 Oct 2013.....