Pulmonary delivery of ISCOMATRIX influenza vaccine induces both systemic and mucosal immunity with antigen dose sparing

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Using a large animal model, we evaluated whether delivery of influenza vaccine via its mucosal site of infection could improve vaccine effectiveness. Unexpectedly, pulmonary immunization with extremely low antigen doses (0.04 μg influenza) induced serum antibody levels equivalent to those resulting from a current human vaccine equivalent (15 μg unadjuvanted influenza, subcutaneously) and vastly superior lung mucosal antibodies. Induction of this potent response following lung vaccination was dependent on addition of ISCOMATRIX adjuvant and deep lung delivery. Functional antibody activity, marked by hemagglutination inhibition, was only present in the lungs of animals that received adjuvanted vaccine via the lungs, suggesting this approach could potentially translate to improved protection. The 375-fold reduction in antigen dose and improved mucosal antibody responses, compared to the current vaccine, suggests that mucosal delivery via the pulmonary route may be particularly relevant in the event of an influenza pandemic, when vaccine supplies are unlikely to meet demand.

INTRODUCTION

Influenza is a major global health issue, characterized by an acute, frequently severe, respiratory illness. Such infections are a significant cause of morbidity and mortality, particularly in the very young and the elderly.[1] In the USA alone, influenza infections are associated with an average of 36,000 deaths and 114,000 hospitalizations each year.[2]

An effective protective vaccine remains the best and most cost-effective strategy for minimizing the impact of influenza. The human influenza vaccine typically comprises 15 μg of antigen from each of the three most prevalent circulating strains of virus, delivered by deep subcutaneous injection without adjuvant. The effectiveness of this vaccine varies significantly, depending on age and immunocompetence of the recipient, and the degree of similarity between viral strains included in the vaccine and those that circulate during the influenza season. With a good match, the influenza vaccine can be 70–90% effective at preventing illness or death in healthy persons < 65 years old.[3] Although the current vaccine is highly beneficial, a more efficacious vaccine would be extremely valuable at further reducing the morbidity and mortality associated with influenza infections.

It is now generally accepted that mucosal surfaces are linked by an integrated immune system, and that immune responses generated at mucosal surfaces are best induced by vaccination at these same sites.[4] Despite this, the vast majority of approved vaccines are still delivered by injection and induce predominantly systemic immunity, even when targeting mucosal pathogens, such as in the case of influenza.

The potential for utilizing the lung as a route of vaccine delivery has been relatively neglected. In particular, although vaccination via the nasal route has proven effective with live attenuated influenza vaccine (best exemplified by the Flumist vaccine), little attention has been paid to the potential of immunizing with killed influenza vaccine, at the mucosal site of infection, the airway mucosa, as a means to improve vaccine effectiveness. During an influenza epidemic in 1967–1968, Waldman *et al.*[5] found that recipients of a bivalent, inactivated influenza vaccine, sprayed into the nasal cavity presented with 79% fewer illnesses than unvaccinated controls. In contrast, only 27% less illness was observed in those injected subcutaneously with the same vaccine. Although this demonstrated that a respiratory delivered vaccine can be safe for humans and potentially more effective than current injected vaccines, it was not possible to

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determine whether this protection was mediated via delivery to the nasal lymphoid tissues, lung inhalation, or a combination of both. Injection of antigen or DNA vaccines into the trachea of mice has been shown to induce both systemic and mucosal immune responses.[6] However, the large doses used may also have stimulated nasal mucosal immune tissue or, if swallowed, stimulated responses via gastrointestinal mucosal immune sites. Moreover, adult mice, unlike healthy humans, can possess organized bronchial-associated lymphoid tissue.[7] Therefore, it is difficult to extrapolate observations made in mice following vaccine delivery by the respiratory route to humans.

Further, women who received bronchial vaccination with a human papillomavirus type 16 virus-like particle produced serum antibody responses comparable to injected vaccine.[8] Similarly, bronchial delivery of a polysaccharide vaccine against pneumococcal infection induced serum antibody responses in both healthy volunteers and patients with chronic obstructive pulmonary disease[9,10] and aerosol delivery of tetanus toxoid-induced serum antibody levels comparable to those induced by injection.[11] It is notable that in all these studies, the vaccines tested did not contain an adjuvant.

Hence, there appears to be considerable potential for the lung as a route of vaccination against influenza, but proper controlled studies are lacking. In this study, we evaluated whether influenza immunization via the lung could improve vaccine effectiveness. Sheep were selected for this study, because they possess lungs of similar size, physiology, and mucosal immune systems to humans.[12–15] Additionally, we examined the effect of adding ISCOMATRIX adjuvant, already proven highly effective at inducing both antibody and cell-mediated responses in sheep and humans.[16]

RESULTS

Pulmonary vaccination with reducing doses of influenza antigen plus adjuvant

In the first experiment, we examined the systemic and mucosal antibody responses following pulmonary immunization of sheep with either 15, 5, or 1 μg H1N1 influenza antigen plus 100 μg of ISCOMATRIX adjuvant. The dose of adjuvant used was similar to that demonstrated in clinical trials to be safe and effective when delivered systemically in humans.[17,18] Control sheep were immunized subcutaneously with 15 μg of influenza antigen, the antigen dose for each influenza strain in current human influenza vaccines. Sera and lung washings, collected before commencement of the experiment and after each immunization, were used to evaluate the antibody responses induced systemically and at the local mucosal site, respectively. The anti-influenza antibody titers in both lung washings and sera were significantly greater in all three groups of sheep immunized by the pulmonary route, compared to sheep that received 15 μg unadjuvanted influenza antigen subcutaneously (**Figure 1**). This observation led to further experimentation where the influenza antigen dose in the ISCOMATRIX vaccine was reduced to 0.008 μg.

Unexpectedly, pulmonary immunization with as little as 0.04 μg influenza antigen plus ISCOMATRIX adjuvant induced serum antibody responses at least equivalent to those induced by 15 μg unadjuvanted antigen delivered subcutaneously, and vastly superior pulmonary antibody levels (**Figure 1**). Antibody responses induced by vaccines containing low antigen doses ($\leqslant 0.2$ μg) delivered by the pulmonary route were slower to develop. Following one dose, only the subcutaneously immunized group and the pulmonary delivered groups that received $\geqslant 1$ μg of antigen had detectable serum immunoglobulin (Ig) G responses (**Figure 1**). After two immunizations, however, the lung delivered formulations induced equivalent serum and mucosal antibodies, even with as little as 0.04 μg antigen. Furthermore, at this antigen dose, three pulmonary immunizations induced superior antibody levels compared with the control group in both sera and lungs (**Figure 1**). For clarity, only IgG data is shown, but essentially the same observations were also made with serum and bronchoalveolar lavage (BAL) IgA responses (data not shown). Importantly, these antibodies had functional activity, even at 0.04 μg antigen, as demonstrated by inhibition of viral agglutination of red blood cells (hemagglutination inhibition (HAI); **Table 1**), the World Health Organization (WHO) approved correlate for protection against influenza.[19] We do not know why no HAI activity was detected in groups receiving 1 μg or 0.2 μg antigen, as this is inconsistent with both the reproducible activity observed with the 0.04 μg antigen dose (see **Table 1** and below) plus their serum and BAL antibody titers. However, it was notable that only animals immunized by the pulmonary route had detectable functional anti-HA activity in BAL samples.

Requirement for both the pulmonary route of delivery and adjuvant for potent immune response to low antigen dose influenza immunization

We further evaluated whether both adjuvant and pulmonary delivery are required to induce strong serum and mucosal antibody responses to low dose influenza vaccines. Sheep were immunized by the pulmonary route or subcutaneously with 0.04 μg influenza antigen plus or minus ISCOMATRIX adjuvant and the anti-influenza antibody responses in sera and BAL assessed. The ability of the low antigen dose vaccine to induce high titer IgG and IgA responses in the sera and lung was dependent on the presence of ISCOMATRIX adjuvant (**Figure 2**). It is worth noting that as BAL samples are already diluted as a consequence of the collection procedure, the actual anti-influenza antibody titers at the pulmonary surface would be even greater than those shown.

Notably, although the subcutaneous low-dose ISCOMATRIX influenza vaccine also induced a good systemic antibody response, it did not induce a lung response comparable to that induced by pulmonary immunization. Both subcutaneous and pulmonary delivery of adjuvanted low-dose vaccines induced serum antibodies with functional activity (as demonstrated by HAI activity) but importantly, however, only sheep immunized via the pulmonary route with adjuvant had functional antibody activity in their lungs (**Table 2**).

The pulmonary immunizations in the above studies were delivered into the left lung. To further characterize the mucosal

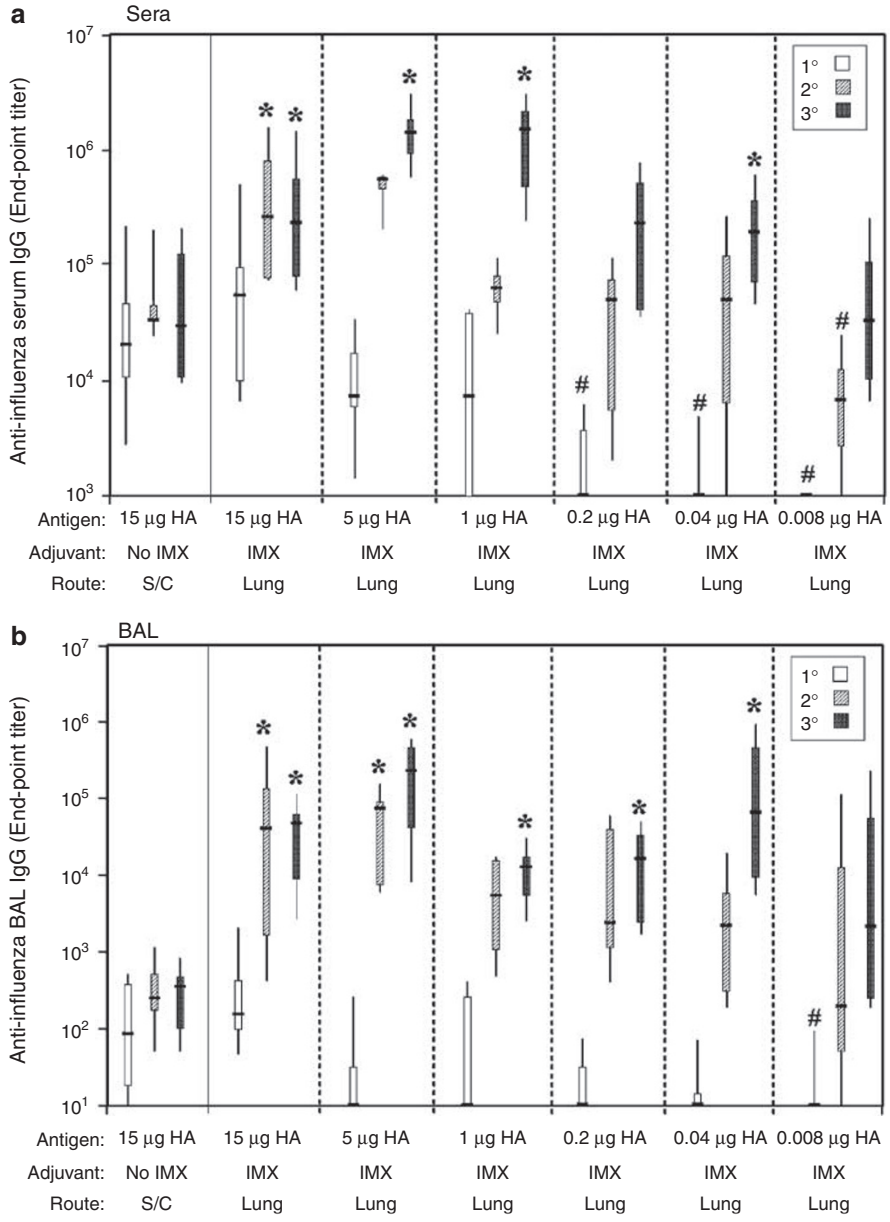

Figure 1 Systemic and mucosal antibody responses induced by reducing doses of ISCOMATRIX (IMX) influenza vaccine delivered by the pulmonary route. Sheep received three immunizations into the caudal lobe of the left lung via bronchoscope. Vaccines were given 3 weeks apart and comprised reducing doses of A/New Caledonian 20/99 H1N1 virus plus 100 μg IMX adjuvant. Positive controls received subcutaneous (S/C) immunizations of 15 μg antigen alone. (**a**) Sera and (**b**) bronchoalveolar lavage (BAL; lung washings) were collected prior to commencement and after each immunization. IgG anti-influenza antibody titers were determined by ELISA and pre-immunization antibody levels subtracted. Data presented are combined from two experiments. All groups contained $n=8$ sheep except the positive control group ($n=12$). Plots present the median titer (horizontal bar), inter-quartile ranges (boxed region) and 10th and 90th percentiles (error bars). 1°, 2°, and 3° present data from primary, secondary, and tertiary vaccinations, respectively. For analysis of significance, data were log transformed and compared by ANOVA with Dunnett's *post hoc* analysis. *Significantly greater than the 15 μg S/C group ($P<0.03$). #Significantly lower than the 15 μg S/C group ($P<0.03$).

immune response to pulmonary delivered influenza vaccine, we also analyzed the antibody responses in the contralateral right lung (a naive site not exposed to influenza antigen). Sheep immunized in the left lung with both influenza antigen and ISCOMATRIX adjuvant had significantly elevated IgG and IgA influenza-specific antibodies present in the BAL from the contralateral right lung, even without local antigen stimulation.

To examine whether pulmonary immunization induced a detectable systemic cellular response, we measured the *in vitro* proliferative response of restimulated peripheral blood mononuclear cells. Peripheral blood mononuclear cells from sheep immunized by the pulmonary route with ISCOMATRIX-adjuvanted low antigen dose vaccine had a detectable cellular response to influenza antigen (**Table 3**). However, no such response was detectable in animals that received low dose antigen alone, demonstrating that a systemic cellular response could be induced with pulmonary delivered low-dose antigen, provided adjuvant was coadministered.

Table 1 Hemagglutination inhibition activity is induced in lung washings by lung immunization with reducing doses of influenza antigen and ISCOMATRIX adjuvant

		HAI titer (geometric mean±s.d.)					
		Serum			Bronchoalveolar lavage		
Route	Vaccine	Primary	Secondary	Tertiary	Primary	Secondary	Tertiary
S/C	15 μg flu	13±2	43±3	95±6	< 10	< 10	< 10
Lung	15 μg flu+IMX	10±0	113±7	95±4	< 10	17±4*	18±3*
Lung	5 μg flu+IMX	< 10	78±4	174±3	< 10	10±1	30±3*
Lung	1 μg flu+IMX	< 10	23±2	147±3	< 10	< 10	< 10
Lung	0.2 μg flu+IMX	< 10	19±2	104±4	< 10	< 10	11±1
Lung	0.04 μg flu+IMX	< 10	21±3	215±4	< 10	< 10	35±4*
Lung	0.008 μg flu+IMX	< 10	11±2	23±3 #	< 10	< 10	12±2

Sheep were immunized three times either subcutaneously (S/C) or via the lungs with influenza antigen (flu) with or without 100 μg ISCOMATRIX adjuvant (IMX). Sera and bronchoalveolar lavage collected after each immunization were analyzed for hemagglutination inhibition activity (HAI). Data, combined from two experiments, are expressed as the titer of sample that inhibited influenza agglutination of red blood cells. Groups contained n=8 sheep except the S/C group (n=12). Preimmunization samples were negative for HAI activity (not shown). For analysis of significance, log-transformed data were compared by ANOVA with Dunnett's post hoc analysis. #Significantly lower than subcutaneously immunized control ($P < 0.03$). *Significantly higher than subcutaneously immunized control ($P < 0.002$).

Comparison of upper and lower lung delivery on the immune response induced by low antigen dose adjuvanted influenza immunization

The above studies were all performed by delivery of vaccines into the deep caudal lobe of the lung. We next evaluated whether deep lung delivery was important for the induction of vaccine-induced mucosal and systemic immune responses. Sheep received three pulmonary immunizations of 0.04 μg influenza ISCOMATRIX vaccine either delivered to the deep caudal lobe (lower lung), or to the upper region of the mainstem bronchi (upper lung). From antibody levels in samples collected 1 week after the third immunization, it is clear that both systemic and mucosal immune responses induced by a low antigen dose adjuvanted influenza vaccine are considerably enhanced when the vaccine is delivered to the deep lung (**Figure 3**). Although anti-influenza antibody levels following deep lung delivery were elevated by 10-fold or less in the sera, the effect was again far more marked in the lung, with an increase of 100- to 1,000-fold compared with upper lung immunization.

DISCUSSION

One of the most serious challenges facing human health today is preparing for the next influenza pandemic. The 1918 pandemic, occurring before the advent of passenger airlines, is estimated to have killed 50 million people.[20] In today's society of rapid global transit, such an infection would likely spread too fast for reactive protective measures to be effective. For this reason many, including the WHO, consider it essential to proactively prepare for such an event.[21] Although an effective vaccine remains the best and most cost-effective strategy for protecting against a pandemic, the WHO estimate that the shortfall in vaccine supply will be in the billions of doses.[21] This shortage could be exacerbated if the next pandemic arises from the H5N1 avian influenza, as antigen yield from reverse-genetics-derived H5N1 virus is only 30–40% that of current vaccine strains.[22] Strategies to address this potential shortfall in vaccine supply are urgently needed. Furthermore, there are issues regarding the efficacy of current influenza vaccines, with the effectiveness of immunizations varying from 26 to 76%, depending on the age of recipient and the match between vaccine and circulating influenza strains.[23]

In this study, we demonstrated that a considerable reduction in influenza antigen dose can be achieved by delivering the vaccine by the pulmonary route. The accepted correlate of protection against influenza is serum IgG with HAI activity ≥ 40. By pulmonary immunization, we achieved equivalent serum antibodies and functional antibody activity to the current vaccine equivalent, with a 375-fold reduction in antigen. Moreover, we induced a far superior lung mucosal antibody response (both IgA and IgG) at the site of potential infection. Unfortunately, sheep cannot be readily infected with influenza, so no challenge study was possible. Further studies showed that this potent effect was dependent on both delivery to the deep lung and the addition of exogenous adjuvant (ISCOMATRIX adjuvant). It was notable that the improved immune response induced by vaccination via the lung required several doses to develop, at least in naive sheep. Given that the majority of humans have either been infected with, or immunized against influenza, it will be important in future studies to evaluate the response in primed animals, to examine the requirement of multiple doses in individuals with memory to influenza. Interestingly, our data parallel a 1969 human study. Waldman *et al.*[24] found that although a single injected vaccination with an A2/Hong Kong vaccine was superior in protecting against influenza compared with an aerosol delivered vaccine, protection induced by two doses via these routes was similar. Our data suggest that had they delivered a third dose, they may have observed greater protection via aerosol vaccination than following injection.

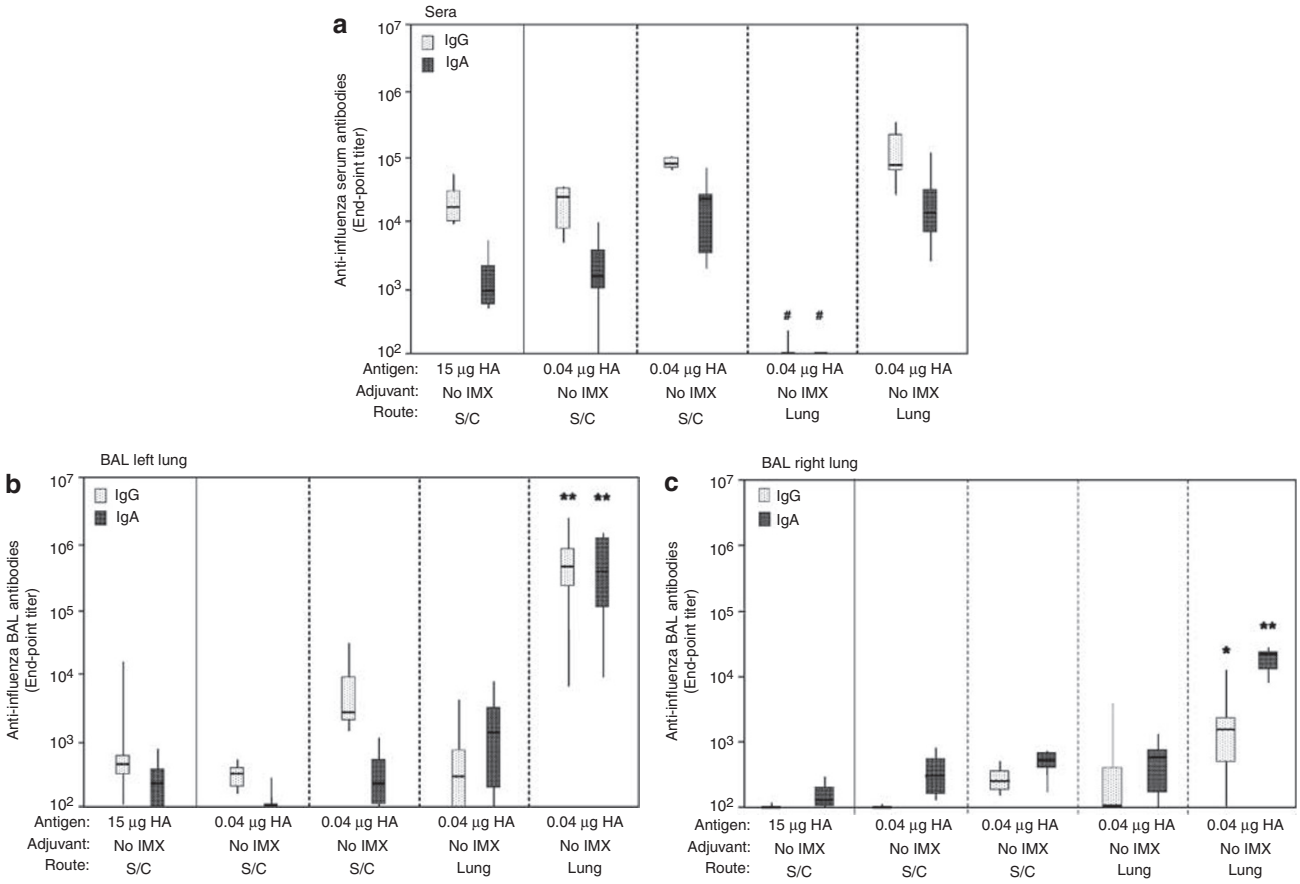

Figure 2 Induction of systemic and mucosal antibodies by immunization with extremely low doses of influenza antigen is dependent on both adjuvant and pulmonary delivery. In a single experiment, sheep (n=8) were immunized three times with 0.04 μg influenza antigen (HA), with or without 100 μg ISCOMATRIX adjuvant (IMX), delivered either via the lungs or subcutaneously (S/C). Positive controls (n=8) received 15 μg antigen alone, S/C. Samples were collected after the third immunization, and IgA and IgG anti-influenza antibody titers determined by ELISA with pre-immunization antibody levels subtracted. (**a**) Serum antibody response; (**b**) lung washings (BAL) from the left lung where vaccine was delivered; (**c**) BAL from the contralateral right lung that was not directly exposed to antigen. Plots present the median titer (horizontal bar), interquartile ranges (boxed region) and 10th and 90th percentiles (error bars). For significance, data were log transformed and compared by ANOVA with Dunnett's *post hoc* analysis. There was no significant difference between titers in sheep vaccinated subcutaneously with 0.04 μg antigen (with or without IMX adjuvant) and the positive controls. #Significantly lower antibody levels compared with all other groups (P<0.001). *Significantly higher antibody levels compared with control group (P<0.01). **Significantly higher antibody levels compared with all other groups (P<0.02).

The requirement to deliver to the deep lung does provide some technological challenges. Although relatively neglected as a route of vaccination, the lung has attracted much attention as a route for drug delivery and it is well documented that deep lung delivery is also required for maximal drug absorption.[25] Research on drug delivery via the lung, such as insulin for the treatment of diabetes, has resulted in considerable advances in technologies required for deep lung delivery. This includes improved delivery devices, optimization of flow rates for lung distribution, and production of engineered particles[26–28] Hence, we are technologically well positioned for translating these observations to humans by delivering vaccine formulations in a controlled manner to the deep lung.

Although subcutaneous injection of low-dose influenza with ISCOMATRIX vaccine induced strong systemic antibodies, sheep immunized by the pulmonary route with the adjuvanted influenza vaccines also had vastly superior mucosal antibody levels with functional activity in their lungs. Increased secretion of influenza-specific mucosal antibodies with functional activity could improve vaccine efficacy by improved neutralization of influenza at the site of infection. Consistent with this, a WHO report stated that mucosal IgA was associated with protection against influenza challenge in mice, and in studies with live attenuated influenza vaccines in man, the presence of mucosal IgA correlated with reduction in virus shedding and resistance to experimental infection.[29] They concluded that mucosal IgA appears to play a role in protection against influenza and more studies should evaluate the ability of vaccines to induce mucosal immune responses.[29] One notable feature of our data is the very high levels of mucosal IgA antibodies induced in the lungs of pulmonary immunized animals, with levels typically increased 100- to 1,000-fold compared to the same vaccine delivered subcutaneously. As influenza is spread directly from person–person via exhalation of virally loaded droplets

Table 2 Hemagglutination inhibition activity in the lungs is only induced by immunization with extremely low doses of influenza antigen when delivered via the lung with adjuvant

		HAI titer (geometric mean±s.d.)					
		Serum			Bronchoalveolar lavage		
Route	Vaccine	Primary	Secondary	Tertiary	Primary	Secondary	Tertiary
S/C	15 μg flu	< 10	43±3	40±3	< 10	< 10	< 10
S/C	0.04 μg flu	< 10	19±2	23±3	< 10	< 10	< 10
S/C	0.04 μg flu+IMX	16±2*,§	320±2*,§	123±3§	< 10	< 10	< 10
Lung	0.04 μg flu	< 10	< 10#	< 10#	< 10	< 10	< 10
Lung	0.04 μg flu+IMX	< 10	13±2#	122±4§	13±2	13±2	55±4*,§

In a single experiment, sheep (n=8) were immunized three times either subcutaneously (S/C) or via the lungs with influenza antigen (flu) with or without 100 μg ISCOMATRIX adjuvant (IMX). Control sheep (n=8) received 15 μg influenza antigen S/C. Sera and bronchoalveolar lavage were collected after each immunization and analyzed for hemagglutination inhibition activity (HAI). Data are expressed as the titer of sample that inhibited influenza agglutination of red blood cells. Pre-immunization samples were negative for HAI activity (not shown). For analysis of significance, data were log transformed and compared by ANOVA with Dunnett's *post hoc* analysis. #Significantly lower than subcutaneous control ($P < 0.03$). *Significantly higher than subcutaneous control ($P < 0.002$). §Significantly higher than group receiving unadjuvanted antigen via the same route of delivery ($P < 0.04$).

Table 3 Pulmonary immunization with ISCOMATRIX adjuvanted influenza vaccine induces antigen-specific memory proliferative responses in peripheral blood mononuclear cells

Pulmonary immunization		Median Stimulation Index (interquartile range) following restimulation with	
Vaccine	Group size	5 μg influenza antigen	10 μg influenza antigen
0.04 μg influenza	n=8	1 (1–2)	1 (1–3)
0.04 μg influenza+ 100 μg IMX	n=8	6 (2–16)	10 (3–25)

Sheep were immunized in their lungs with either 0.04 μg influenza antigen alone, or 0.04 μg influenza+100 μg ISCOMATRIX adjuvant. 1 week after the third immunization, peripheral blood was collected, and mononuclear cells cultured in 96-well plates with either media alone (control), or 5 or 10 μg/ml influenza antigen (restimulated). After 4 days, wells were pulsed with tritiated thymidine for 24 h. Radioactivity was measured and Stimulation Indices calculated by dividing the mean counts per minute (c.p.m.) for the restimulated group by the mean c.p.m. for the control group. A clear systemic antigen recall proliferative response was evident in sheep immunized via the pulmonary route with antigen plus adjuvant, but not with antigen alone.

that are inhaled by uninfected individuals, the production of effective neutralizing antibodies in the lung may also reduce transmission and therefore limit the spread of infection during a pandemic. Reduction in transmission is likely to be of particular importance in a pandemic situation, as early on it is likely that a large percentage of the population will be unvaccinated. Thus, a vaccine that reduces transmission may be more successful in protecting a large population than subcutaneous vaccines, which require very high rates of vaccination (>80%) to establish significant herd immunity.[30]

The specific mechanism by which deep lung delivery of this vaccine formulation induces the observed immune response is unknown. Substances in the deep lung can traverse the epithelium and enter the bloodstream, to induce systemic antibody and cellular immune responses. However, as a significant proportion of subcutaneously delivered vaccine would also enter the blood, either directly or via lymphatic drainage, this is unlikely to explain the enhancement in mucosal antibodies compared with injected vaccine. This suggests there are unique elements associated within the deep lung responsible for inducing the mucosal component of the immune response. One candidate is the alveolar macrophage, which is the predominant cell type located within the mucosal lumen of the deep lung. These phagocytic cells could potentially act as antigen-presenting cells and/or secrete cytokines that instigate the mucosal response to vaccine antigens. Similarly, lung dendritic cells are also an obvious candidate for inducers of this response.[31] These cells are likely to drain to local lymph nodes where the induction of mucosal immune responses may occur. Further work is required to dissect these precise mechanisms.

A major unmet need is the lack of an effective mucosal adjuvant suitable for use in humans. Hence, the demonstration of the potent activity of an influenza ISCOMATRIX vaccine when delivered via a mucosal route may be a highly significant observation. The ability of ISCOMATRIX vaccines delivered by the pulmonary route to induce responses at distant mucosal sites is currently being investigated. ISCOMATRIX adjuvant has shown great promise, thus far proven both safe and highly immunostimulatory in clinical trials when delivered systemically[17,18,32] Furthermore, in preliminary safety studies, pulmonary delivery of ISCOMATRIX adjuvant in sheep, even at dose levels 50-fold in excess of the anticipated human dose, have been shown to be safe (unpublished observation). The data presented in this study suggest that further exploration of this technology as a mucosal adjuvant is warranted.

In summary, pulmonary delivery of an ISCOMATRIX influenza vaccine appears to have considerable advantages. It allows the use of very low levels of antigen, while inducing a systemic antibody response equivalent to the current vaccine plus, most significantly, a far superior mucosal antibody response. The

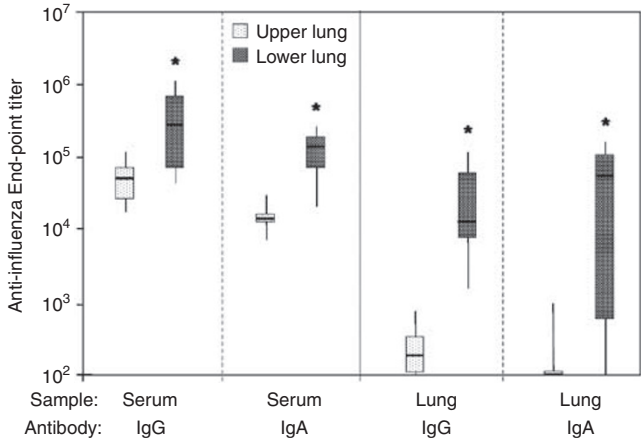

Figure 3 Comparison of upper and lower lung low-dose influenza immunization on induction of systemic and mucosal antibody responses. In a single experiment, sheep ($n=8$) were immunized three times with 0.04 μg influenza antigen (HA), plus 100 μg ISCOMATRIX adjuvant, delivered either to the upper (about 2 cm past the major bifurcation between the left and right lungs) or lower (segmental bronchi in the caudal lobe) left lung. Sera and left lung washings were collected after the third immunization, and IgA and IgG anti-influenza antibody titers determined by ELISA, with preimmunization antibody levels subtracted. Plots present the median titer (horizontal bar), interquartile ranges (boxed region) and 10th and 90th percentiles (error bars). *Significantly higher in sheep immunized via the lower lung, compared with the upper lung ($P<0.05$).

main benefit of the low antigen requirements would be realized in the event of an influenza pandemic when shortages in vaccine supply are currently inevitable. In addition, the greater induction of local functional antibodies provides the possibility for improved vaccine efficacy. This could translate both as reduced morbidity and mortality to influenza infection, as well as improved herd immunity by minimizing the potential spread of the virus from infected vaccinated individuals.

MATERIALS AND METHODS

Animals

Female Merino ewes were housed in pens within the School of Veterinary Science animal facility, The University of Melbourne, Parkville. Sheep were fed lucerne chaff mixed with commercial pellets and allowed access to water *ad libitum*. All experimental procedures were approved by the Veterinary Science Animal Experimentation Ethics Committee at the University of Melbourne.

Pulmonary and subcutaneous vaccinations

Influenza antigen was sucrose gradient purified A/New Caledonian 20/99 H1N1 virus, which had been inactivated and detergent disrupted. Antigen concentration was based on hemagglutinin content and determined by single radial immunodiffusion. ISCOMATRIX adjuvant consists of typically 40 nm cage large structures, comprised of phospholipid, cholesterol, and purified saponin. GMP grade ISCOMATRIX adjuvant was prepared by CSL Limited as previously described.[16]

For standard pulmonary immunizations, sheep were carefully restrained in a harness and a bronchoscope (Pentax 16FG), lubricated with a lignocain gel, inserted via the nostril to the lower caudal lobe of the lung. Vaccines were infused in a total volume of 5 ml, followed by 10 ml of air to ensure complete delivery. For subcutaneous immunizations, vaccines were delivered in a total volume of 200 μl in the inner thigh. Immunizations were spaced by 3 weeks. Where required, sera and lung washings were collected 1 week prior to commencement of the experiment, 2 weeks (primary response) after the first immunization and 1 week (memory response) after secondary and subsequent immunizations.

Blood (10 ml) was collected from the jugular vein using an 18-gauge needle with syringe, then left to coagulate for collection of sera. For collection of lung washings (BAL), 10 ml of phosphate-buffered saline was delivered into the caudal lobe of the lung via a bronchoscope and then immediately withdrawn. Samples were stored at -20 °C until analyzed.

Evaluation of antibody responses by ELISA

Anti-influenza antibodies in BAL and serum samples were evaluated by ELISA. Briefly, 96 well Maxisorp flat bottom plates (NUNC, Roskilde, Denmark) were coated overnight with 50 μl of 10 μg/ml influenza antigen in carbonate buffer, pH 9.6. Plates were then blocked with 1% sodium casein before adding 100 μl of 1 in 5 serial dilutions of samples in duplicates. Binding of specific anti-influenza antibodies was detected using rabbit anti-sheep IgG conjugated with horseradish peroxidase (SouthernBiotech, Birmingham, AL) or anti-bovine/ovine IgA (Serotec, Oxford, UK) followed by rabbit anti-mouse horseradish peroxidase (Dako, Glostrup, Denmark). Color was developed by addition of TMB substrate (Zymed, San Francisco, CA), stopped by addition of 2 M H_2SO_4, optical density at 450 nm determined on a Bio-Tek ELx800 plate reader and endpoint titers calculated.

Evaluation of hemagglutination inhibition activity

The HAI assay determines the titer of functional antibodies by measuring the inhibition of red blood cell agglutination by influenza virus. Serum and BAL samples were tested for HAI against egg grown A/New Caledonia/20/99 virus (H1N1) using turkey red blood cells according to the method of Kendal.[33] The HAI titer was determined as the dilution of sample that inhibited influenza agglutination of red blood cells. HAI assays were performed by the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia.

Peripheral blood mononuclear cell antigen stimulation proliferation assay

Blood collected by venipuncture into sterile tubes containing heparin was centrifuged, the buffy coat collected and centrifuged again over Ficoll-paque Plus (Amersham Biosciences, Buckinghamshire, UK). Peripheral blood mononuclear cells were collected from the interface. Peripheral blood mononuclear cells (5×10^5 per well) were plated into 96 flat-well plates in complete Dulbecco's

modified Eagle's medium (Gibco, Grand Island, NY) plus 10% fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 50 μM 2-Mercaptoethanol (Sigma, St. Louis, MO). Influenza H1N1 antigen was added to triplicate wells at final concentrations of either 0, 5, or 10 µg/ml and cells cultured for 4 days before overnight pulsing with 1 μCi per well of tritiated thymidine. Using a Packard Harvester, cells were collected onto glass fiber filters, which were placed into 96-well microplates before addition of 25 μl per well Microscint O (PerkinElmer, Boston, MA). Thymidine uptake was measured using an automated microplate scintillation counter.

Statistical analyses

For all statistical analyses, data were log transformed and compared by one-way analysis of variance with Dunnett's *post hoc* analysis using SPSS software, version 13.0.

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DISCLOSURE

Data presented in this paper was used in a patent application. SE, MP, JPS, and PS were named inventors on this patent application.

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