CD71 targeting boosts immunogenicity of sublingually delivered influenza haemagglutinin antigen and protects against viral challenge in mice.

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Short Title: Transferrin conjugation boosts sublingual immunogenicity

17

18 **Abstract**

19 The delivery of vaccines to the sublingual mucosa is an attractive prospect due to the ease
20 and acceptability of such an approach. However, novel adjuvant and delivery approaches are
21 required to optimally vaccinate at this site. We have previously shown that conjugation of
22 protein antigen to the iron transport molecule, transferrin, can significantly enhance mucosal
23 immune responses. We tested whether conjugating influenza haemagglutinin to transferrin
24 could improve the immune response to sublingually delivered antigen. Transferrin conjugated
25 haemagglutinin induced a significant antibody and T cell response in both naïve animals and
26 previously immunized animals. The immune response generated was able to protect mice
27 against influenza virus challenge. Sublingually administered antigen dispersed more widely
28 through the gastro-intestinal tract than intranasally delivered antigen and transferrin
29 conjugation had a more marked effect on sublingually delivered antigen than intranasal
30 immunisation. From these studies we conclude that transferrin conjugation of antigen is
31 effective at boosting immune responses to sublingually delivered antigen and may be an
32 attractive approach for influenza vaccines, particularly when mass campaigns are required.

33

34 **Introduction**

35 Mucosal surfaces are commonly exploited as portals of entry to the body by a wide range of
36 invasive pathogens including the influenza virus, a common cause of respiratory viral
37 infection [1, 2]. Influenza targets the upper respiratory tract and in some cases can infect the
38 lower lungs. Influenza infections are thought to affect between 5-15 % of the world's human
39 population, resulting in 3-5 million cases of severe illness and 250,000-500,000 deaths
40 annually [3]. Vaccination against influenza is still widely regarded as the best form of
41 defence against both seasonal and pandemic influenza. Vaccination rates are highly variable
42 across European countries, from <5 % in Estonia to >75 % in the Netherlands in the >55 age
43 group [4], thus improving the ease of delivery of influenza vaccines may improve uptake and
44 therefore reduce the global burden of disease.

45 One approach to improve vaccine delivery is to use alternate routes of delivery and oral
46 vaccination has a number of advantages compared to conventional parenteral delivery or
47 other mucosal routes. Oral vaccination removes the potential infection risk associated with
48 needle stick injuries to hospital staff or the reuse of contaminated needles in the developing
49 world setting and the acceptability of oral drops under the tongue compared to needles is high
50 [5]. Furthermore, in urgent situations where mass vaccination needs to be achieved in
51 relatively short periods of time, oral vaccination could allow less qualified healthcare workers
52 to carry out immunization campaigns [6].

53 The sublingual route of oral vaccine delivery has long been investigated due to its numerous
54 antigen sampling points and organized lymphoid structures [7-10]. However, it has had
55 limited success in larger animals [11] and therefore strategies to boost efficacy are required.

56 Here we evaluate transferrin (Tf), the CD71 targeting ligand, for its ability to be utilized as a
57 conjugate vaccine strategy capable of targeted delivery and entry to mucosal tissues. Tf is a
58 reversible iron binding protein that is transported into cells by Tf receptor mediated
59 endocytosis. The Tf receptor (CD71) has previously been exploited for oral insulin drug
60 delivery in rats [12] and we have previously shown that Tf could significantly augment
61 immune responses against a model trimeric HIV-1 clade C immunogen, gp140 when
62 administered via the nasal route [13].

63 Here we show that through coupling of haemagglutinin (HA) antigen to Tf, the resulting
64 conjugate vaccine when delivered sublingually, resulted in significant concentrations of
65 antigen-specific serum IgG and systemic T cell responses. This induced response was
66 associated with a significant reduction in infection-associated weight loss as well as reduced
67 virus titres in the lungs. Therefore, Tf conjugation could be an effective tool to improve
68 sublingual antigen delivery.

69

70 **Results**71 *Conjugation of transferrin to haemagglutinin significantly enhances antibody responses to*
72 *sublingually administered antigen.*

73 Sublingual delivery of antigen has been shown to elicit potent immune responses and we
74 have previously demonstrated that biochemical coupling of human holo-Tf protein to
75 candidate vaccine antigens facilitated receptor mediated trans-epithelial transport and
76 significantly enhanced immune responses of topically applied, mucosally delivered, vaccine
77 formulations [13]. We conjugated holo-Tf to haemagglutinin (HA) derived from the H3N2
78 Influenza (X31 strain) using a 3:1 molar ratio of Tf-biotin:HA-streptavidin to achieve
79 efficient conjugation without excess Tf remaining in the reaction mixture (data not shown).
80 The immune response to Tf-HA vaccine formulation was compared to unconjugated HA in
81 female BALB/c mice immunized three times, at two week intervals with 20 µg protein
82 applied topically to the sublingual mucosa in a 30 µl volume. Sublingual delivery of HA
83 induced an HA specific IgG response in the sera, but Tf conjugation significantly enhanced
84 the response (** $p < 0.01$, **Fig. 1a**).

85 Due to the high prevalence of circulating virus, vaccinated individuals are unlikely to be
86 influenza naïve and we therefore wanted to explore the impact of Tf-HA in the context of
87 pre-existing immunity. To test this, we evaluated whether Tf-HA could boost responses after
88 sublingual priming with a DNA vaccine expressing HA. In previous studies, we have
89 demonstrated that HA delivered as DNA can induce a potent immune response [14] and have
90 demonstrated that mucosal DNA delivery can be potentiated by PEI [10]. Mice were
91 immunized three times with DNA sublingually and subsequently boosted with HA alone or
92 Tf-HA. The DNA + Tf-HA group elicited significantly higher levels of IgG (7525 ng/ml)

than the DNA alone group (734 ng/ml, $**p < 0.01$) or the DNA + HA group (1612 ng/ml, $*p < 0.05$, **Fig. 1b**). Furthermore, DNA + Tf-HA induced a similar response to three immunisations of Tf-HA alone. Antigen-specific IgA was not detected in sera from immunized animals. Taken together these data demonstrate that Tf conjugation can significantly enhance antibody responses to sublingually delivered influenza HA protein, even in the context of pre-existing immunity.

Figure 1. Conjugation to transferrin boosts antibody responses to influenza HA. (A) Female BALB/c mice (n=8 per group) were vaccinated by sublingual administration 3 times at 2 week intervals with either 10 µg Tf-HA or 10 µg HA alone. (B) Alternatively mice (n=12) were immunized sublingually with 20 µg DNA plasmid expressing HA complexed with deacetylated PEI three times prior to a single 10 µg sublingual protein vaccination. Antigen-specific serum IgG antibody responses were measured at the end of the study, week 10.

As part of vaccine safety profiling, it is important to evaluate the immunogenicity of carrier proteins in any vaccination regimen. Thus, it is noteworthy that we have previously evaluated anti-transferrin humoral immunity in vaccinated animals as an indicator of potential adverse immune responses. Importantly, we observed a complete absence of any induced anti-Tf antibodies [13], suggesting Tf conjugation is safe and did not cause a breach in central tolerance. Critically, no overt toxicity, as evidenced by altered behavioral activity, decreased food and water consumption, weight loss, wheezing, piloerection or diarrhea, was witnessed at any time during the vaccination process.

Conjugation of transferrin to haemagglutinin significantly enhances the elicited T cell response to sublingual antigen.

One drawback of current influenza immunisation programs is the necessity for seasonal vaccination due to the drift in circulating strains. Cellular responses to influenza vaccines have been shown to be important in cross-protective immunity [15]. To assess vaccine elicited T cell responses, we performed IFN- γ ELISpot on isolated splenocytes using HA whole antigen stimulation 3 weeks after the final vaccination. Vaccination with Tf conjugated HA elicited significantly higher IFN- γ T cell responses than unconjugated HA protein (** $p < 0.01$, **Fig. 2a**). In mice that had previously been immunized 3x with DNA expressing HA, subsequent sublingual HA protein immunisation significantly boosted the T cell response, and conjugation with Tf did not significantly increase the response compared to protein alone (** $p < 0.01$, *** $p < 0.001$, **Fig. 2b**). Interestingly we found the Tf-HA alone regimen, without any prior DNA-HA priming, to be equivalent to both the DNA + Tf-HA and the DNA + HA prime-boost regimens with respect to the elicited T cell responses. This suggests that conjugation of Tf to HA can efficiently prime T cell responses when administered via mucosal surfaces. Heterologous prime-boost vaccination has previously been used to

synergistically amplify T dependent antibody responses to vaccinating antigens, with DNA-priming facilitating memory B cell development, which is then selectively amplified upon recombinant protein-boost vaccination [16]. The similarity in T cell responses measured between the DNA + HA and DNA + Tf-HA regimens can most likely be attributed to the DNA-priming vaccine. However the differences seen in antibody titer (Fig. 1) between these groups is most probably due to the increased bioavailability of topically applied Tf-HA to antigen presenting cells within the body compared to HA alone (which has no receptor targeted entry).

Figure 2. Conjugation to transferrin boosts T cell responses to influenza HA. (A) Female BALB/c mice (n=7 per group) were vaccinated by sublingual administration 3 times at 2 week

intervals with either 10 µg Tf-HA or 10 µg HA alone. **(B)** Alternatively mice were immunized sublingually with 20 µg DNA plasmid expressing HA complexed with deacetylated PEI, three times prior to a single 10 µg sublingual protein vaccination. IFN γ cellular responses in splenocytes to influenza HA protein were measured at the end of the study, week 10.

Conjugation of transferrin to haemagglutinin conveys protection against Influenza challenge infection.

To assess the effect of Tf conjugation on protein delivered topically to the sublingual mucosae we used an influenza infection model. Immunized mice were intranasally infected with 50 haemagglutination units/ml X31 (strain A/X-31 H3N2) and weight loss assessed as a correlate of disease. Sublingual immunization with Tf-HA induced significant levels of protection compared to naïve animals ($*p = 0.031$, **Fig. 3a**) and Tf-HA-boost of DNA immunized animals also gave significantly greater protection than the DNA + HA regime or untreated animals (**Fig. 3a**). Using a plaque assay to assess influenza infection in the lungs of vaccinated mice at day 4 following infection we found the heterologous DNA + Tf-HA and triple Tf-HA vaccination regimens significantly reduced the recoverable, viable virus titres in lung samples compared to negative control mice ($***p = 0.0006$ and $**p = 0.0012$, **Fig. 3b**). These data indicate that Tf conjugation can enhance protection against infection. This is further supported by the observation that DNA + Tf-HA treated mice had significantly reduced viral titres in their lungs compared to DNA + HA vaccinated mice ($*p < 0.05$, **Fig. 3b**). Intriguingly, the DNA + HA group also had significantly reduced viral titres compared to naïve animals, in spite of similar disease profiles ($*p < 0.05$, **Fig. 3b**).

We further evaluated the ability of Tf-HA compared to unconjugated HA to convey protection using a high dose challenge model, using 1.25×10^6 /ml PFU X31 influenza. Mice were infected 1 week after the last sublingual vaccination. Here we found the DNA prime,

168 Tf-HA boost treatment regimen reduced the measurable infection-associated weight loss
169 (median value = 92.2%) compared to the DNA + HA (median value = 86.7%) treatment
170 regimen by day 5 post infection (**Fig. 3c**). Furthermore it became very evident that the Tf-HA
171 treatment group had a lower level of infection associated morbidity compared to the HA
172 regimen. Mice were culled when weight loss exceeded 20% as a humane endpoint. Critically,
173 the mortality rates between the DNA + Tf-HA regimen and the DNA + HA regimen were
174 considerably different with only 16.6% of animals in the DNA + Tf-HA regimen compared to
175 50% of mice in the DNA + HA regimen reaching their humane endpoint after infection (**Fig.**
176 **3d**). Taken together, this clearly demonstrates that the biochemical conjugation of Tf to HA
177 vaccine antigen increased the protective efficacy of the vaccine formulation by reducing both
178 morbidity and mortality against a robust challenge infection.

Figure 3. Conjugation to transferrin boosts protection against influenza virus challenge. Female BALB/c mice (n=7 per group) were prime vaccinated by sublingual administration 3 times at 2 week intervals with 20 μ g of DNA-PEI polyplexes expressing HA prior to a single sublingual 10 μ g Tf-HA or HA alone protein vaccination. Mice were subsequently infected with 100 μ l influenza X31 virus intranasally. (A) weight loss was recorded daily and (B) viral load was measured on day 4 after infection by plaque assay. In a second study mice were challenged with a high dose of influenza (MLD₅₀), with (C) weight loss recorded over 14 days and (D) mortality and recovery assessed.

Transferrin conjugation is essential to elicit antigen-specific immunity via the sublingual route

221 Next, we used protein alone as previously we have demonstrated that Tf conjugation
222 improves the efficacy of nasally delivered HIVgp140. We wished to compare nasal and
223 sublingual delivery of influenza HA antigen and the effect on protection against challenge.
224 Female BALB/c mice were immunized three times, at two week intervals with 20 µg protein
225 applied topically to the nasal or sublingual mucosa. HA delivered alone sublingually elicited
226 a poor antibody response, but Tf conjugation induced significantly greater levels of specific
227 antibody (** $p = 0.0079$, **Fig. 4a**). Interestingly HA alone delivered intranasally induced an
228 anti-HA response (** $p = 0.0079$), which was further boosted by Tf conjugation (** $p =$
229 0.0079). To test whether mucosal immunization was protective, mice were challenged
230 intranasally with X31 virus. Sublingually applied Tf-HA was able to positively influence
231 disease outcome by significantly reducing disease associated weight loss compared to naïve
232 controls (** $p = 0.0079$) and the unconjugated HA treatment regime (** $p = 0.0079$) on days 5
233 and 6 after infection (**Fig. 4b**). Both HA (** $p = 0.0079$) alone or Tf-HA (** $p = 0.0079$)
234 treatment regimens delivered intranasally protected against viral infection on days 5 and 6
235 post challenge.

Figure 4: Transferrin conjugation has a significantly greater effect on antigen delivered sublingually in contrast to the intranasal route. (A) Female BALB/c mice (n=5 per group) were vaccinated 3 times at 2 week intervals with either Tf-HA or HA alone via the sublingual (SL) or intranasal (IN) routes. Antigen-specific IgG antibody responses were recorded at the end of the study, week 10. (B) Mice were infected with X31, body weights for the different treatment groups were recorded daily to monitor infection associated weight loss and are expressed as a percentage relative to day 0. Statistical significance was assessed using Mann Whitney U test with $*p \leq 0.05$ or $**p \leq 0.005$.

We wished to determine the distribution of Tf conjugated antigen after delivery by different routes. To localize antigen after intranasal or sublingual delivery, we used fluorescently labelled Tf (**Fig. 5**). Fluorescent signal was detectable in the nasal/oral cavity at 10, 20, 30, 60 and 120 minutes after either route of delivery. To investigate Tf distribution in the nares, lung, liver, stomach and small intestine, mice were culled at various times after application and dissected to examine these internal organs (**Fig. 5a**). Interestingly, we found that labelled

252 Tf administered SL had reached the stomach and small intestine within 10 min with only
253 small quantities being detectable within the lung. The oral cavity retained the formulation for
254 a short period of up to a maximum of one hour. In contrast the formulation applied IN was
255 measured at high levels in the lung within 10 min and persisted in that organ for at least 2
256 hours. The nasal associated lymphoid tissue (NALT) within the nares exhibited a strong
257 signal at all timepoints examined, indicating that IN exposure to antigen, perhaps
258 unsurprisingly, persisted for a much longer time than oral administration due to natural
259 swallowing and libation by the animal. Within 30 min the distribution pattern in the stomach
260 and small intestine had equalised between the two different administration routes, with only
261 the IN applied formulation still being observed in the lung and NALT tissue (Fig. 5b). This
262 rapid transport of the formulation to the gut after oral administration, and the slightly delayed
263 but still rapid appearance of IN applied formulation in the gut indicates that the potentiating
264 activity of the transferrin on the immune response likely occurs within the oral cavity, due to
265 increased transport of the antigen into local blood vessels and lymph nodes.

266

267 **Figure 5. Sublingually and Intranasally delivered formulations exhibit different organ**
 268 **distribution patterns.** *In vivo* distribution of fluorescent Tf-Alexa 670 after topical administration to
 269 sublingual or nasal mucosa. (A) 100 μ g Tf-Alexa in a 20 μ l volume of PBS was applied topically to
 270 the murine nasal cavity or sublingual space. Fluorescent signal was acquired with a 4 second exposure
 271 through a 670 nm filter. Images were taken at 10 minutes post delivery. Live animals were maintained
 272 under anesthetic during imaging, animals were culled before organ removal for analysis. Lg = Lung,
 273 Lr = Liver, S = Stomach, S.I = Small Intestine. (B) Quantification of the fluorescence intensity within
 274 each organ. Organs were removed and the relative intensity of the fluorescence specific to each organ
 275 measured and graphed.

Discussion

Collectively, these and our previous studies show that Tf protein can act as an enhancer of conjugated antigen uptake at the mucosal surface and potentiate humoral immune reactivity towards vaccine antigens. This increased antigen uptake activity of Tf is not restricted to the nasal route of delivery and is seen after oral delivery of vaccinating antigens. Conjugation to Tf significantly improved the efficacy of sublingually delivered antigen and the effect was more marked with sublingual delivery than intranasal. Intranasal delivery of vaccinating antigens can elicit immune responses in the nasal associated lymphoid tissues (NALT) and in the lungs, while sublingual application has been shown to mediate immune responses via the sublingual mucosa and the gut associated lymphoid tissue (GALT) [17-19]. It may be that because influenza targets respiratory surfaces, inducing a local response in the respiratory tract gives superior protection compared to responses in the GI tract, though this requires further investigation. Alternatively, it may be that the respiratory tract in mice is more sensitive to foreign antigen, indeed in previous studies we have observed a greater response to intranasal than sublingual delivery of both HIV and tetanus antigens [20]. However, there are possible issues around the safety of intranasal immunisation, especially when inflammatory adjuvants are used [21], which may make the sublingual route more attractive.

In the current study, we observed that sublingually applied antigen did not remain in the buccal cavity, but was distributed throughout the GI tract within 30 minutes of administration. This was in marked contrast to intranasal delivered antigen, which was retained in the nasal mucosa and the lung, though a major proportion also ended up in the stomach and intestines. This is likely a biomechanical issue, with animals swallowing material from the mouth more readily than from the nose and also due to the relatively high volume administered sublingually in contrast to previously published studies where volumes

less than 10 ul are employed to ensure the antigen dose remains in the buccal cavity [22, 23]. There may also be more rapid uptake of Tf in the nose, and we have previously observed high CD71 expression on nasal epithelia [13], because good control of freely available iron is a key innate defence mechanism against bacterial infection [24]. This rapid distribution of antigen may in part explain the difference in efficacy of unconjugated antigen in the two routes, as orally delivered antigen has a poor response due to either digestion by the GI system proteases, sequestration in gastric mucus and dilution by the large liquid volumes moving throughout the intestines [25]. It is clear that the transferrin targeting of antigen significantly improves immunogenicity but it is not yet clear where the effect occurs, possibly within the short time the material is present within the oral cavity, but transfer of antigen across the mucosal barrier may also happen as the conjugate travels through the upper GI tract and then during it's residency in the small intestine where there is a high density of Peyer's patches.

An important feature to consider in the development of oral vaccines is how to overcome the oral tolerogenic barrier. Classical experiments have demonstrated that administration of soluble antigens via the oral route can result in unresponsiveness to subsequent systemic challenge with the same protein [13] [26]. While it is thought that tolerance is the default pathway for most mucosal tissues, breaking the tolerogenic pathway can convey vaccine-mediated protection against infection. Examples of licensed oral vaccines include Sabin live oral polio vaccine, live oral rotavirus vaccine and the live oral typhoid vaccine. Inactivated and attenuated vaccines could potentially enhance the immunogenicity of the vaccine formulation and overcome oral tolerance by expressing pathogen associated molecular patterns which could engage with host cell pro-inflammatory Toll-like receptors (TLRs). The dominant mechanism behind oral tolerance is induction of Treg cells. Such cells are known to impede Th1 and Th17 cell responses and result in reduced IFN- γ secretion. We

therefore believe that we were able to subvert oral tolerance using transferrin conjugated hemagglutinin as evidenced by production of B cell class switched antigen-specific IgG and strong IFN- γ T cell responses. [27]. A factor, other than increased bioavailability of antigen, which could have contributed towards overcoming tolerance is the presence of streptavidin within our conjugate vaccine. Although not measured in this study, streptavidin and streptavidin conjugates can elicit anti-streptavidin antibodies and can feasibly act as an adjuvant in promoting the immunogenicity of the vaccine formulations, after the vaccine cargo crosses the mucosal surface [28]. Therefore it is possible that in addition to the CD71 transport capabilities, the presence of streptavidin in the vaccine formulation further enhanced the immunogenic nature of the formulation.

Annual influenza vaccination has been recommended for adults aged ≥ 55 years and certain high-risk groups for decades. Repeat vaccinations with new antigen formulations are essential for protection against diseases caused by organisms displaying a high degree of genetic variability. Within this manuscript we present data concerning multiple oral vaccinations and demonstrate protection against challenge infection. However, while the authors acknowledge that multiple vaccinations within a single influenza season may be difficult to implement, the advantages of oral immunization are clear. These advantages include being less painful as they do not require administration using needle and syringe, greater degrees of compliance, ease and rapidity of administration – not requiring medically trained personnel to inject the vaccine and reducing the risk of needle stick infections [29]. Critically, oral vaccines prime mucosal immune responses, which can be of significant protective benefit at mucosal front line surfaces. Future planned studies will evaluate whether the years following the initial oral vaccination will require multiple immunizations, or whether a single boost with the new strains hemagglutinin will be sufficient.

One of the issues with influenza vaccination is that the vaccine needs to be effective in the context of pre-existing immunity due to the high prevalence of circulating viruses. We assessed the effect of Tf-HA compared to sublingual HA alone in animals that had previously been immunized with DNA vaccines. DNA vaccines are a promising technology that are highly effective in small animal models, but have failed to deliver on this promise in clinical trials [30]. However, they can provide a useful priming immunisation, especially in the induction of T cells which have been proposed to be an important component of a universal influenza vaccine that is cross-protective against multiple strains [15].

Conclusion

Within this study we have used a low dose of topically applied antigen to test our mucosal targeting system. The observation that concentrations of antigen as low as 10 µg/dose can mediate protection signifies that Tf is efficient at delivering antigen via the oral route. We are optimistic that future planned studies involving the administration of larger concentrations of Tf-HA, in smaller volumes, may increase the elicited systemic antibody response against HA and thus further increase protection. Furthermore, it would be interesting to co-formulate Tf-HA with molecular adjuvants such as TLR agonists [20, 31-33] to further boost immune reactivity. In conclusion we provide further evidence that Tf can be utilized as a mucosal adjuvant, capable of augmenting immune responses to biochemically coupled vaccine antigens. To confirm translatability of these observations, these investigations will need to be performed in humans or larger animals.

Materials and Methods

Influenza virus and haemagglutinin antigens.

The MDCK cultured X31 (a recombinant strain of H3N2/A/Aichi/2/1968 surface proteins and A/Puerto Rico/8/1934 internal proteins) was generously provided by Dr Andreas Wack (The Crick Institute, UK). The vaccinating trimeric X31 haemagglutinin, obtained by bromelain treatment of virus, was provided by Prof John McCauley (The Crick Institute, UK) as a 5 mg/ml solution and the recombinant X31 haemagglutinin antigen (A/Aichi/2/1968) used in our assays was from Sino Biological Inc. (China).

Transferrin-haemagglutinin chemical conjugation.

Influenza haemagglutinin was modified using LL-modifier reagent before being reacted in a 1:1 molar ratio with Streptavidin using a Lightning-Link Streptavidin conjugation kit (Innova Biosciences, UK), as described previously [13]. The reaction was quenched using the LL-quench and the protein incubated for 30min to ensure the reaction had terminated. Biotinylated human holo-Tf (Sigma Aldrich, UK) was then added to the haemagglutinin-streptavidin conjugate and the reaction incubated for 1 hour. Verification of successful conjugation was carried out by both protein gel electrophoresis and western blot using serum from mice which had previously resolved homologous influenza infection or using anti-human holo-Tf antibody (Sigma, UK). All vaccine formulations were made immediately prior to immunizations.

Mice, vaccinations, sampling and infections.

Female BALB/c mice (Harlan, Glasgow, UK), 6-8 weeks old, were placed into groups of n=5 and housed in a fully acclimatized room. All animals were handled and procedures performed in accordance with the terms of a project license granted under the UK Home Office after approval by a local Animal welfare and ethics board. Animals (Scientific Procedures) Act 1986. Food and water were supplied *ad libitum*. All mice received either three topically

applied fortnightly sublingual vaccinations of 10 µg (low dose) or 25 µg (high dose) HA or Tf-HA vaccinating antigen in a volume of 20 µl, or intranasal immunisation of HA and Tf-HA antigen in a volume of 30 µl. For experiments utilizing DNA sublingual prime vaccinations, mice were administered with 20 µg sublingual plasmid DNA complexed PEI (Polysciences, Ger) using a PEI to DNA ratio of 4:1. The plasmid DNA encoded influenza X31-HA cloned into the pmaxFP™-Red C vector (Lonza, UK) as described previously [10].

Tail bleeds were collected weekly without anti-coagulant and centrifuged in a Heraeus Biofuge pico (Fisher, UK) at 1000 g for 20 min. The serum was harvested and transferred into fresh 0.5 ml micro-centrifuge tubes (Starlab, UK), and stored at -20°C until antibody titres were determined by ELISA. For IFN-γ ELISpot analysis, lymphocyte cultures were derived from single cell suspensions from spleens of immunized and control mice as previously described [33].

For low antigen dose influenza infectious challenge studies, Influenza X31 virus stocks at 500 Haemagglutination units /ml were diluted to 50 Haemagglutination units /ml in PBS and 100 µl administered nasally to anaesthetized mice. Mice body weights were monitored every day for productive infection as determined by weight loss. To determine lung viral loads, mice were culled 4 days post infection. For high dose studies, female, 8-week-old BALB/c mice were administered a 50% mouse lethal dose (MLD₅₀). To calculate the MLD₅₀, mice were anesthetized with isoflurane and intranasally inoculated using 5-fold serial dilutions (3.13x10⁷/ml – 5x10⁴/ml) of X-31 influenza viruses in 100 µl PBS. Animals that showed signs of severe disease and weight loss of ≥20% were withdrawn from the study and euthanized.

Antigen-specific Semi-Quantitative ELISA

A quantitative Influenza HA-specific ELISA was followed. Briefly, ELISA plates were coated with 100 µl per well of 5 µg/ml Influenza HA antigen diluted in sterile PBS. Plates were then covered with a plate seal and incubated overnight at 4°C. The plates were then washed four times by PBS-T (Invitrogen, UK) and blocked by adding 200 µl per well of assay buffer (1 % BSA in 0.05 % PBS-T). The plates were then sealed and incubated for 1 hour at 37°C. After the incubation, the plates are washed 4 times with 250 µl of PBS-T. A 50µl volume of sample diluted 1:1000, 1:10,000 and 1:50,000 in sample diluent were then added to the plate in triplicate. Plates were then resealed and incubated for 1 hour at 37°C. The plates were washed 4 times with 250 µl of PBS-T per well, before the addition of a 1:4000 dilution in sample diluent of 100 µl/well of either anti-mouse IgG-HRP, anti-mouse IgG1-HRP, anti-mouse IgG2a-HRP or IgA-HRP (Southern Biotech). Plates were incubated for 1 hour at 37 °C before 4 washes with 250 µl PBS-T. Plates were developed using the addition of 100 µl/well TMB and the reaction stopped after 5min using Stop solution (Insight Biotechnologies, UK). Standards on the plate consisted of coating with 100 µl anti-mouse Kappa (1:3200) and Lambda (1:3200) light chain (Serotec, UK), blocking as before and then adding 50 µl of IgG (Southern Biotech, UK) or IgA (Southern Biotech, UK) in a 1:3 dilution down the plate starting at 200 ng/ml. The rest of the process was the same as for the samples where the secondary antibodies were incubated for 1 hour, washed, the reaction developed with TMB and stopped with Stop solution (Insight Biotechnologies, UK). Mucosal samples were assayed in duplicates using a 1:10, 1:50 and 1:250 dilution. For mucosal IgA, biotinylated-IgA (1:4000) was used followed by one hour incubation with Streptavidin-HRP (R&D systems). The absorbance was read on a KC4 Spectrophotometer at 450 nm (Bio-Tek Industries, Inc).

Antigen-specific Endpoint ELISA

Antigen-specific haemagglutinin binding antibody titres were measured using a standardised ELISA. MaxiSorp high binding 96-well plates were coated with 50 µl HA at 5 µg/ml in PBS for 1 hour at 37°C. Plates were washed three times in PBS-T before blocking with 200 µl PBS-T containing 10% foetal bovine serum for 1 hour at 37°C. After further washing, sera diluted 1:100 or mucosal wash samples diluted 1:10 were added at 50 µl /well and incubated for 1 hour at 37°C. Plates were washed four times before the addition of 50 µl of either a 1:10,000 dilution of goat anti-mouse IgG-HRP or a 1/2000 dilution of biotinylated anti-mouse IgA (all Southern Biotech, UK) secondary antibody and incubated for 1 hour at 37°C. The plates were washed four times and developed with 50 µl/well of KPL SureBlue TMB substrate (Insight Biotechnology, UK). The IgA required a further Streptavidin-HRP (R&D systems) amplification step prior to TMB development. The reaction was stopped after 15min by adding 50 µl/well 1 M H₂SO₄, and the absorbance read at 450 nm on a KC4 spectrophotometer.

Assessment of vaccine elicited T cell responses by IFN-γ ELISpot

For assessment of IFN-γ T cell responses in splenocyte cultures, 15 µg/ml AN18 coating antibody was used to coat ELISpot plates (Millipore, UK). After washing and blocking, 5 µg/ml of Influenza HA was used to stimulate the cells to assess antigen-specific responses. Unstimulated and 5 µg/ml PHA (Sigma, UK) polyclonal activator stimulated cells served as controls for each sample. To detect spots, biotinylated anti-IFN-γ was added at 1 µg/ml for 2 hours before washing and incubating with Streptavidin-ALP for 1 hour. Plates were washed as before and 100 µl/well of either BCIP/NBT substrate added, allowed to develop before washing with tap water, dried and read using the AID ELISpot reader ELR03 (Autoimmun Diagnostika GmbH, Ger) and the AID ELISpot READER 4 software (Autoimmun Diagnostika GmbH, Ger). Background was calculated as twice the mean of negative controls.

Positive samples were determined as exceeding 50 spot forming units (SFU)/million cells after subtraction of background.

Determination of lung viral titres

Lung virus samples were attained by excising infected lungs and then disrupting them using 2ml PBS and 70 µm Nylon Cell Strainers (BD Falcon, UK). The lung preparations were then centrifuged at 350 g for 10 min. The supernatants were decanted and frozen at -20°C. To measure lung viral loads, 90% confluent MDCK cell cultures were grown in a 24 well plate using complete DMEM (10% FCS, 2mM L-glutamine, 100 U/ml penicillin and 1% non-essential amino acids). Infected lung samples were defrosted and incubated on ice prior to adding to PBS washed MDCK cells. Lung samples were added either, neat, diluted 1:10, 1:100 or 1:1000 in incomplete DMEM for 1 hour at 37°C. Viral inoculums were removed, cells washed with PBS before adding 2ml overlay medium to each well (7.5ml of 2% agarose supplemented with 2.3µg/ml trypsin and 17.5ml incomplete DMEM). Plates were incubated for 3 days at 37°C before counting plaques.

In vivo fluorescent imaging.

Real time bio-imaging of orally applied fluorescently labelled Tf was assessed using a multispectral Carestream *In Vivo* FX Pro system (USA). A volume of 20 µl of Tf-Alexa (647nm) was applied topically to the sublingual space or the nares of anaesthetised female BALB/c mice. Three mice were used per timepoint (10, 20, 30, 60, 120 and 300 min) with two or three experimental repeats at each time. Imaging was carried out immediately prior to and at the various timepoints after application. Photonic emissions were captured using white light with a 0.25 second exposure and after a 4 and 10 second multi-wavelength exposure

489 with a 670nm filter and images acquired and analysed using the supplied Carestream
490 software (USA).

491 ***Statistical analysis.***

492 Statistical analysis of the data was carried out using a nonparametric Mann–Whitney, *U*-test
493 and using GraphPad PRISM software.

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Figure Legends

Figure 1. Conjugation to transferrin boosts antibody responses to influenza HA. (A)

Female BALB/c mice (n=8 per group) were vaccinated by sublingual administration 3 times at 2 week intervals with either 10 µg Tf-HA or 10 µg HA alone. **(B)** Alternatively mice (n=12) were immunized sublingually with 20 µg DNA plasmid expressing HA complexed with deacetylated PEI three times prior to a single 10 µg sublingual protein vaccination. Antigen-specific serum IgG antibody responses were measured at the end of the study, week 10.

Figure 2. Conjugation to transferrin boosts T cell responses to influenza HA. (A)

Female BALB/c mice (n=7 per group) were vaccinated by sublingual administration 3 times at 2 week intervals with either 10 µg Tf-HA or 10 µg HA alone. **(B)** Alternatively mice were immunized sublingually with 20 µg DNA plasmid expressing HA complexed with deacetylated PEI, three times prior to a single 10 µg sublingual protein vaccination. IFNγ

cellular responses in splenocytes to influenza HA protein were measured at the end of the study, week 10.

Figure 3. Conjugation to transferrin boosts protection against influenza virus challenge.

Female BALB/c mice (n=7 per group) were prime vaccinated by sublingual administration 3 times at 2 week intervals with 20 µg of DNA-PEI polyplexes expressing HA prior to a single sublingual 10 µg Tf-HA or HA alone protein vaccination. Mice were subsequently infected with 100 µl influenza X31 virus intranasally. (A) weight loss was recorded daily and (B) viral load was measured on day 4 after infection by plaque assay. In a second study mice were challenged with a high dose of influenza (MLD₅₀), with (C) weight loss recorded over 14 days and (D) mortality and recovery assessed.

Figure 4: Transferrin conjugation has a significantly greater effect on antigen delivered sublingually in contrast to the intranasal route.

(A) Female BALB/c mice (n=5 per group) were vaccinated 3 times at 2 week intervals with either Tf-HA or HA alone via the sublingual (SL) or intranasal (IN) routes. Antigen-specific IgG antibody responses were recorded at the end of the study, week 10. (B) Mice were infected with X31, body weights for the different treatment groups were recorded daily to monitor infection associated weight loss and are expressed as a percentage relative to day 0. Statistical significance was assessed using Mann Whitney U test with $*p \leq 0.05$ or $**p \leq 0.005$.

Figure 5. Sublingually and Intranasally delivered formulations exhibit different organ distribution patterns.

In vivo distribution of fluorescent Tf-Alexa 670 after topical administration to sublingual or nasal mucosa. (A) 100 µg Tf-Alexa in a 20 µl volume of PBS was applied topically to the murine nasal cavity or sublingual space. Fluorescent signal was acquired with a 4 second exposure through a 670 nm filter. Images were taken at 10 minutes post delivery. Live animals were maintained under anesthetic during imaging, animals were

656 culled before organ removal for analysis. Lg = Lung, Lr = Liver, S = Stomach, S.I = Small
657 Intestine. **(B)** Quantification of the fluorescence intensity within each organ. Organs were
658 removed and the relative intensity of the fluorescence specific to each organ measured and
659 graphed.

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