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1 Influenza vaccine: Engineered nucleoprotein with improved protective efficacy against
2 multiple strains
3
4 Circulating influenza A virus (IAV) strains differ in their surface proteins each year, and
5 vaccines eliciting an immune response to these proteins are often only partially protective.
6 Internal viral proteins, such as the nucleoprotein (NP), are highly conserved, and cellular
7 immunity to NP has been correlated with protection from diverse strains. However, current
8 IAV vaccines induce a poor immune response to NP. In this study, led by Fergal Hill from
9 Osivax, researchers develop an oligomeric version of NP with improved immunogenicity.
10 Vaccination of mice with oligomeric NP results in an improved NP-specific T cell response,
11 including CD8⁺ tissue memory T cells in the lung, and protects mice against three different
12 IAV subtypes. Co-administration with the currently used inactivated influenza vaccine
13 further improves protection against virus infection in mice. These results encourage further
14 preclinical and clinical development for this vaccine candidate.
15
16

1 **OVX836 a recombinant nucleoprotein vaccine inducing cellular responses and**
2 **protective efficacy against multiple influenza A subtypes**

3 **Running Title: OVX836 a broad-spectrum influenza A recombinant vaccine**

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29 **Abstract**

30 Inactivated influenza vaccines (IIV) lack broad efficacy. Cellular immunity to a
31 conserved internal antigen, the nucleoprotein (NP), has been correlated to protection
32 against pandemic and seasonal influenza and thus could have the potential to broaden
33 vaccine efficacy. We developed OVX836, a recombinant protein vaccine based on an
34 oligomerized NP, that shows increased uptake by dendritic cells and immunogenicity
35 compared to NP. Intramuscular immunization in mice with OVX836 induced strong
36 NP specific CD4+ and CD8+ T cell systemic responses and established CD8+ tissue
37 memory T cells in the lung parenchyma. Strikingly, OVX836 protected mice against
38 viral challenge with three different influenza A subtypes, isolated several decades
39 apart and induced a reduction in viral load. When co-administered with IIV, OVX836
40 was even more effective in reducing lung viral load.

41

42 **Keywords:** Influenza vaccine, recombinant Nucleoprotein, oligomerized
43 Nucleoprotein, cellular immunity; CD8+T cells response, protection, efficacy,
44 immunogenicity, broad-spectrum, Influenza A, pandemic, seasonal.

45 **Introduction**

46 Inactivated influenza vaccines aim to prevent infection, and are usually partially
47 successful. A recent meta-analysis from 2004-2015 seasons found an efficacy of 33%
48 (confidence interval (CI) = 26%–39%) against H3N2 viruses, compared with 61% (CI
49 = 57%–65%) against H1N1 and 54% (CI = 46%–61%) against influenza B viruses ¹.

50 During the pH1N1 pandemic of 2009, monovalent vaccines containing an adjuvant
51 had median effectiveness of 69% (CI=60-93%) ¹. But by targeting only the surface
52 glycoproteins, current influenza vaccines run the risk of having much lower
53 effectiveness should a mismatch occur between the strains included in the vaccine and
54 the strains actually circulating. This happened most recently in the Northern
55 Hemisphere during the 2014-2015 season ², in which vaccine effectiveness was
56 estimated to be as low as 23% ³, due to drift mutations in the hemagglutinin antigenic
57 site B of the circulating H3 strain ⁴, but current influenza vaccines retain some
58 effectiveness even when mismatches occur ⁵.

59 Nonetheless, a vaccine approach based on cellular responses to conserved internal
60 antigens could minimize the effects of antigenic drift and even mismatches. Influenza
61 pandemics are very informative in this respect: In a retrospective serological study ⁶
62 of the H2N2 pandemic of 1957, suggestive evidence was found that adults exposed to
63 the new virus were spared from influenza more frequently than children. The author
64 proposed that the main protective factor in adults was immunity resulting from prior
65 infection with earlier (non-H2N2) strains. The recent H7N9 influenza outbreak, with
66 more than 600 documented cases has been particularly instructive. Indeed, a small
67 study of 16 hospitalized patients lacking neutralizing antibodies showed that those
68 who recovered (12 patients) had significantly stronger CD8+, CD4+ and NK cell
69 immune responses to the H7N9 virus than those (4 patients) who did not recover from

70 the infection ⁷. Noteworthy, early cytotoxic T lymphocyte (CTL) responses were
71 associated with more rapid recovery ⁷.

72 The H1N1 pandemic of 2009 provided further opportunities to determine whether
73 heterosubtypic immunity to influenza exists in humans. A prospective cohort study
74 conducted during the H1N1 pandemic of 2009 showed that individuals who
75 developed less severe illness harbored higher frequencies of pre-existing T cells with
76 specificity for conserved CD8 epitopes ⁸. In human challenge studies, on the other
77 hand, pre-existing CD4+, but not CD8+T cells responding to influenza internal
78 proteins were associated with lower virus shedding and less severe illness ⁹.

79 Most recently, the Flu Watch Cohort Study ¹⁰ has investigated whether pre-existing T
80 cell responses targeting internal viral proteins could provide protective immunity
81 against pandemic and seasonal influenza. NP is one of the most conserved internal
82 influenza antigens; even the most divergent protein sequences differ by less than 11%
83 ¹¹. The presence of NP-specific T cells before exposure to virus correlated with less
84 symptomatic, PCR-positive influenza A, during both pandemic and seasonal periods,
85 whereas other conserved antigens were at best of lesser importance ¹⁰. Additionally, it
86 has been recently possible to demonstrate a strong immune selection against NP
87 CD8+ epitopes in the human influenza lineage when compared to those of the swine
88 lineage. This observation highlights the importance of immune responses against this
89 internal NP antigen, notably considering that other conserved viral antigens, such as
90 the matrix (M1) protein, do not undergo a similar immune selection ¹².

91 Finally, a comprehensive study comparing systematically the conserved influenza
92 antigens, administered in MVA, found that only vectors expressing NP, either alone
93 or in combination with other conserved influenza proteins, protected mice against
94 lethal challenges with H5N1, H7N1 and H9N2 ¹³. Altogether, these studies, added to

95 the fact that NP contains CD8+ epitopes that can be dominant in both HLA-A2-
96 positive ¹⁴ and HLA-A2-negative humans ¹⁵, illustrate the potential utility of cellular
97 immune responses against this conserved NP influenza antigen. NP has been regularly
98 detected in influenza inactivated vaccine (IIV) formulations ^{16, 17}, in variable, but
99 sometimes considerable amounts ¹⁸. However, it has been shown that, when included
100 in the IIV, NP exerts relatively poor CD8+ T cell immunogenicity both in humans
101 and in mice ¹⁹. There is a need, therefore, for novel forms of influenza NP capable of
102 significantly enhancing the immunogenicity of this antigen, even when administered
103 with IIV.

104 Oligomeric forms of protein antigens have been shown to be more immunogenic than
105 the monomeric forms ²⁰. To enhance the immunogenicity of NP, we have developed
106 OVX313, an improved version of the oligomerization domain IMX313 ²¹⁻²⁶ created as
107 a hybrid of the C-terminal fragments of two avian C4bp α chain sequences that
108 naturally oligomerize into heptamers ²². The heptameric oligomerization domain
109 OVX313 is stabilized by intermolecular disulfide bonds and the extremely high
110 oligomer stability is an interesting scaffold for applications in the field of protein
111 engineering and vaccines.

112 This study describes OVX836, a recombinant protein candidate vaccine, which
113 consists of the fusion of OVX313 with the nucleoprotein of influenza A
114 (H1N1/WSN/1933). OVX836 increased immunogenicity over NP and protected
115 against multiple influenza subtypes.

116 **RESULTS**

117 **Design of OVX836, based on NP fused to a small oligomerization domain and**
118 **DNA based immunization**

119 We set out to make a more immunogenic version of NP by fusing it to the small
120 oligomerization domain OVX313, derived from the previously described IMX313^{21,22}
121 and described in Materials and Methods (Figure 1a). Then, to determine whether the
122 immunogenicity of the influenza NP could be improved by fusing it to either IMX313
123 or OVX313, four plasmids were constructed (NP, NP-IMX313, NP-OVX313=
124 OVX836 and empty vector) and used to immunize mice.

125 Cellular immune responses induced two weeks after the last DNA vaccination were
126 measured in spleen by ELISpots using either NP to stimulate total T cells (Figure 1b)
127 and purified CD4⁺ T cells (Figure 1c), or the immunodominant peptide NP₃₆₆₋₃₇₄ to
128 stimulate CD8⁺ T cells (Figure 1c). The administration of NP DNA alone induced a
129 weak IFN- γ producing T cells response, not significantly different from the empty
130 vector. On the contrary, we showed that significant amounts of NP-specific IFN- γ
131 producing T cells, including NP-specific CD4⁺ and CD8⁺ T cells response, were
132 induced by OVX836 DNA vaccination, the level of response being increased
133 compared to the response achieved after empty vector, NP or NP-IMX313 DNA
134 vaccination ($p < 0.01$).

135 In parallel, serum antibody responses to NP were measured 14 days after the last
136 immunization. Mice immunized with NP alone showed only low levels of NP-specific
137 IgG antibody responses, while OVX836 immunized mice had significantly higher IgG
138 antibody responses compared to all other groups of immunized mice ($p < 0.01$) (Figure
139 1d).

140 In summary, our results show that fusing NP to the OVX313 domain improves both

141 NP antigen-specific T cell and IgG antibody responses, with OVX836 showing
142 immunogenicity superior to NP-IMX313, which is already more immunogenic than
143 NP.

144 **OVX836 protein is an oligomeric form of NP with improved immunogenicity**

145 Based on the results obtained with DNA immunizations, two recombinant proteins
146 were produced in *Escherichia coli*: NP from the prototype human isolate A/Wilson-
147 Smith/1933 (ref. 27), referred to here as NP, and the same antigen fused through its
148 C-terminus to OVX313, called OVX836. Both OVX836 and NP proteins were
149 purified using Heparin Sepharose affinity columns. The purity of the recombinant
150 proteins was analyzed by SDS-PAGE under reducing and denaturing conditions
151 (Figure 2a, left). OVX836 and NP resolved as single bands at approximately 64 and
152 56 kDa, as expected from the cloned amino acid sequences (theoretical MWs of 62.7
153 and 56.2 kDa, respectively). Moreover, OVX836 and NP were recognized by a MAb
154 raised against NP (Figure 2a, right).

155 OVX836 and NP were then examined by means of reversed-phase (RP)
156 chromatography, showing that purified recombinant proteins were very homogeneous
157 (Figure 2b). Both molecules exhibited a single elution peak that represents >97% of
158 the total protein area at 280 nm. OVX836 and NP showed a main peak corresponding
159 to the heptameric and monomeric form of NP, respectively. As expected, treatment
160 with the reducing agent TCEP did not modify the chromatographic profile of NP. In
161 contrast, the reduction of disulfide bonds promoted dissociation of the OVX836 sub-
162 units into lower association states (Figure 2b), showing that intermolecular disulphide
163 bonds were required for the stabilization of the heptameric state of OVX836 under RP
164 conditions. Under aqueous conditions, OVX836 is heptameric independently from
165 disulphide bonds.

166 Based on the results from gel filtration chromatography, the most abundant form of
167 NP was a trimer of 150 kDa and only few monomers were observed (Supplementary
168 Figure 1a). The analysis of the oligomeric state of purified recombinant NP has
169 indicated a broad distribution of oligomeric states^{28,29}. In contrast, a mutated version
170 of NP did not self-associate and formed only monomers of 55 kDa as the E339A and
171 R416A mutations in the tail loop result in the complete loss of NP trimerization²⁹.
172 The heptameric state of OVX836 as well as small oligomers of heptamers (di-/tri-
173 heptamers) were consistently observed (MW in a range from 400 to 1200 kDa), that is
174 consistent from batch-to-batch. Interestingly, the OVX836 tail loop mutant did not
175 show small oligomers of heptamers (Supplementary Figure 1b).
176 Therefore, OVX836 and NP are pure and homogenous proteins, and compared to the
177 native NP trimer of 150 kDa, OVX836 is mainly organized as mono-, di- and tri-
178 heptamers under aqueous conditions (400-1200 kDa) (Supplementary Figure 1a). Our
179 experiments also indicated that OVX836 is a stable heptamer due to noncovalent
180 intermolecular interactions as well as the formation of disulfide bridges between the
181 subunits. The Figure 2c shows a schematic representation of OVX836 protein
182 heptamer.
183 We then investigated the mechanisms by which OVX836 could be up-taken by
184 professional antigen-presenting cells such as dendritic cells (DCs). DCs were
185 generated in vitro from murine bone marrow cultivated with Flt3 ligand (Flt3L), as a
186 convenient source of pDC and cDC that are equivalent to the spleen CD8 α ⁺ and
187 CD8 α ⁻ DC³⁰. We first checked whether OVX836 was internalized by the different
188 DCs subset. Imaging using ImageStreamX showed a strong internalization of
189 OVX836, localizing in vesicles within DCs (Supplementary Figure 2a, b). Kinetic
190 studies using flow cytometry using Alexa-647 labelled proteins (3mol dye/mol

191 protein) confirmed that OVX836 and NP products are rapidly internalized (within 2
192 hours) by DC (Figure 2d). The levels of incorporation are dose and time dependent
193 (percentage and MFI), with OVX836 being more efficiently incorporated than NP,
194 even after 24 hours incubation.

195 The immunogenicity of the recombinant proteins NP and OVX836 were measured in
196 C57BL6 mice following two intramuscular (i.m) injections with doubling doses from
197 6.25 µg to 50 µg. Fourteen days after the last immunization immune responses against
198 the NP protein were detected by *ex vivo* IFN-γ ELISPOT to measure NP specific IFN-
199 γ producing T cells in response to NP stimulation (Figure 2e). The results showed that
200 the NP induced moderate T cell immune responses and OVX836 protein was more
201 immunogenic at all doses than NP, reaching a maximum response from 25 µg ($p <$
202 0.0001).

203 Furthermore, OVX836 elicited high titre serum NP specific IgG antibodies, which
204 increased with the dose and were higher than with NP at all doses ($p < 0.01$) (Figure
205 2f). Together, these results demonstrated that the fusion protein OVX836 more
206 effectively stimulates T cells and B cells than NP alone.

207 **OVX836 induces high NP specific systemic immunogenic responses, local T cells**
208 **in the lung, and can be used in combination with IIV**

209 We next studied the effect of OVX836 at 25µg alone, or in association with the IIV in
210 C57BL/6 mice, following two i.m injections; these co-administrations took place in
211 the same hind limb but with injection into different muscles. To characterize
212 the cellular immune responses elicited in mice, NP specific IFN-γ after the two
213 immunizations were quantified using ELISPOT assays. In the group immunized with
214 OVX836 or OVX836 combined with IIV, a strong NP specific IFN-γ producing T
215 cells response was induced in the spleen and in lung. This response was significantly

216 higher than in control group $p < 0.0001$ (Figure 3a-b). IIV as expected elicited only
217 low levels of NP-specific T cell responses. In addition, in both the spleen and lung,
218 OVX836 and the combination of OVX836 with IIV by the i.m route were highly
219 immunogenic, inducing NP-specific CD4⁺ and NP₃₆₆₋₃₇₄-specific CD8⁺ T cells
220 response more than ten-fold higher than PBS group (Figure 3c-d).

221 To additionally characterize the NP-specific immune response induced by OVX836,
222 we assessed the magnitude of cytokines released in the culture supernatants from the
223 lung cells stimulated with NP₃₆₆₋₃₇₄ peptide, using an ELISA (Mabtech).
224 Immunization with OVX836 alone or in combination with IIV induced significantly
225 higher levels of IFN- γ , TNF- α and IL-2 compared to control PBS or IIV group (but
226 not IL-4) (Supplementary Figure 3) . These data suggest that OVX836 was able to
227 induce a strong Th1 immune response in the lung with significant increases in the
228 production of IFN- γ , TNF- α and IL-2 cytokines that can contribute to influenza
229 clearance.

230 Specific CD8⁺ T cell response in the lung and spleen by ELISpot, was induced by
231 OVX836, suggesting that the antigen was cross presented. We also analyzed the
232 quality of the CD8⁺ T cells induced by OVX836 alone or in combination with IIV.
233 The frequency of NP₃₆₆₋₃₇₄-specific CD8⁺ T cells following immunization was
234 assessed using pentamer staining by flow cytometry (Figure 3e, f) 14 days after the
235 last immunization to confirm the CD8⁺ T cell activation. Within the spleens of mice,
236 we observed an increased percentage of NP₃₆₆₋₃₇₄-specific CD8⁺ T cells following
237 immunization with OVX836 alone or in combination with IIV compared to IIV or to
238 unvaccinated groups (Figure 3e). We next analyzed the presence of NP₃₆₆₋₃₇₄-specific
239 memory CD8⁺ T cells within the lung parenchyma using intravascular staining to
240 exclude cells in the lung vasculature. Immunization with OVX836 induced the

241 generation of NP₃₆₆₋₃₇₄-specific memory CD8⁺ T cells that were located within the
242 lung parenchyma (Figures 3f). Interestingly, the combination of OVX836 with IIV
243 elicited even higher numbers of lung resident memory CD8⁺ T cells ($p < 0.05$). These
244 results indicate that OVX836 induced virus-specific memory CD8⁺ T cells that can
245 infiltrate lung tissues.

246 Taken together, our results demonstrate that OVX836 induces strong systemic NP-
247 specific CD4⁺ and CD8⁺ T cells responses as well as NP-specific IgG; it also induces
248 lung memory CD8⁺T cells against NP localized in the lung parenchyma.

249 **Vaccination with OVX836 protects mice against lethal infection with the**
250 **influenza H1N1 A/WSN/33 strain**

251 The most important feature of an effective vaccine candidate is the capacity to protect
252 individuals against symptoms and signs of infection. To evaluate this aspect, we
253 performed experimental infections in C57BL/6 mice previously immunized with
254 either PBS (control), IIV, OVX836, or a combination of IIV and OVX836
255 (OVX836/IIV), using the H1N1 A/WSN/33 influenza virus, harboring the same NP
256 protein present in OVX836. In order to eliminate possible model-associated bias in
257 the interpretation of results and anticipating further pre-clinical evaluation, identical
258 experiments were also performed using the other classic influenza infection mouse
259 model, Balb/c mice. As shown in Figure 4, intranasal (i.n.) inoculation of PBS-
260 immunized (control) mice with H1N1 A/WSN/33 influenza strain resulted in 90%
261 mortality and 20% maximum mean weight loss in both mouse strains. On the other
262 hand, whereas immunization with IIV conferred partial protection (60% survival
263 rates) against the H1N1 A/WSN/33 challenge (not included in the IIV formulation) in
264 Balb/c, it failed to protect C57BL/6 mice (10% survival rate). More importantly, both
265 OVX836 and OVX836/IIV vaccinated groups were highly protected against viral

266 challenge in both mouse strains, as shown by survival rates of 90% and 100% in
267 C57BL/6 and 100% and 100% in Balb/c mice, respectively (Figure 4a, b).
268 Additionally, although a mild weight loss was observed in Balb/c mice immunized
269 with OVX836 (10% maximum mean weight loss (Figure 4d), no significant weight
270 loss (<5%) was observed for either the OVX836 or OVX836/IIV immunized in
271 C57BL/6 (Figure 4c). OVX836 and OVX836/IIV vaccination also induced 1-log or
272 higher reductions in lung viral titers (LVTs) compared to the PBS control group,
273 especially in the case of the OX836/IIV combination (Figure 4e, f). Finally, both pre-
274 and post-challenge HAI titers against the H1N1 A/WSN/33 virus as well as the viral
275 strains contained in the IIV formulation were comparable between the two mouse
276 strains, hence validating the IgG responses expected as a result of both IIV
277 vaccination and challenge (Table 1). Of note, OVX836 vaccination did not induce any
278 HAI responses, and tended to increase the HAI response toward some viral strains
279 included in the IIV.

280 These results demonstrate that OVX836 immunization protected both C57BL/6 and
281 Balb/c mice against a lethal challenge infection with H1N1 A/WSN/33 strain.
282 Moreover, when IIV and OVX836 were co-administered, decreased viral load in
283 lungs were observed.

284 **Vaccination with OVX836 induces broad protection against lethal challenge with**
285 **different influenza A subtypes.**

286 In view of the positive results obtained for H1N1 A/WSN/33 infection in two
287 different mouse strains, we further investigated the protective capacity of OVX836
288 against two additional influenza subtypes A, in the Balb/c lethal infection model
289 (Figure 5). Mice were immunized with either PBS, IIV, OVX836, or a combination of
290 IIV and OVX836 (OVX836/IIV) following the same experimental protocol described

291 above, and then i.n. infected with either H1N1 A/California/7/2009 (homologous to
292 the H1N1 strain present in the IIV and containing an NP protein with 93% identity to
293 the OVX836 NP protein) or H3N2 A/Victoria/3/75 (heterologous to IIV and
294 containing an NP protein with 90% identity to the OVX836 NP protein) viruses. Of
295 note, preliminary experiments were performed in order to calibrate the viral inoculum
296 and lung sampling time-point to obtain comparable mortality rates among the
297 different viral strains used as well as to measure LVTs at the peak of viral replication.
298 Infection of PBS-immunized (control) mice with H1N1 A/California/7/2009 or H3N2
299 A/Victoria/3/75 resulted in 100% and 90% mortality, respectively, as well as >20%
300 maximum mean weight loss in both cases (Figure 5a-b). As expected, the two IIV-
301 containing groups (IIV and OVX836/IIV) showed no signs of mortality or weight loss
302 following infection with the H1N1 A/California/7/2009 virus, which in turn produced
303 mild (>10%) weight loss in the OVX836 group with 100% survival rate (Figure 5a,
304 cC). This observation is further supported by 1-log, 4-log and 6-log reductions in
305 mean LVTs for the OVX836, IIV and OVX836/IIV groups, respectively, compared to
306 the PBS control group (Figure 5e).

307 IIV conferred only partial protection (40% survival and 18% weight loss) against the
308 non-homologous H3N2 A/Victoria/3/75 virus. Conversely, both OVX836 and
309 OVX836/IIV vaccinated groups were fully protected (100% survival) against lethal
310 viral challenge with the H3N2 influenza A strain, yet showing 15% mean maximum
311 weight losses. Indeed, the main difference for these two groups in terms of weight
312 loss was driven by a shorter recovery time compared to the PBS and IIV groups
313 (Figure 5b, d). OVX836 and OVX836/IIV vaccination induced 1-log and 2-log
314 reductions in mean LVTs, respectively (Figure 5f). Once again, HAI titers against the
315 viral strains used for infection as well those included in the IIV vaccine formulation

316 confirm the expected antibodies response following IIV vaccinations and challenge
317 (Supplementary Table 1). Interestingly, pre-challenge H1N1 A/California/7/2009 HA
318 titers induced by the OVX836/IIV group tended to be higher than those of the IIV
319 group alone and OVX836 vaccination did not induce any HAI responses, consistent
320 with the observations of Table 1.

321 Taken together, the results of the different challenge tests demonstrate the efficacy of
322 OVX836 alone to provide protection against lethal viral infection with different
323 influenza A strains. In addition, when OVX836 and IIV were combined for the
324 vaccination, IIV being either homologous or heterologous to the viral strain used for
325 the challenge, decrease of the viral loads in lungs were achieved.

326 **DISCUSSION**

327 Influenza is one of the major infectious disease threats for humans in terms of annual
328 health impact of seasonal influenza and in terms of potential dramatic global
329 consequences of an influenza pandemic outbreak ³¹. Licensed influenza vaccines
330 induce antibodies against the hemagglutinin, a highly polymorphic surface antigen,
331 and as such, need to be reformulated and re-administered every year. A vaccine
332 providing protective immunity against highly conserved, mostly internal, antigens
333 could provide longer-lasting protection and cover multiple influenza subtypes ^{32, 33}.
334 However, even though such universal influenza vaccines are subject to much
335 discussions and many reviews, only a handful of candidates have reached the clinical
336 testing stage ^{34, 35}. Despite their limited efficacy ³⁴, current seasonal vaccines remain
337 the most effective way to prevent influenza outbreaks and annual vaccination is
338 recommended for all people aged ≥ 6 months in the United States and Europe ³⁶⁻³⁸.
339 A wide consensus developed the hypothesis that NP probably plays a role in influenza
340 protection with the induction of NP-specific CD8⁺ T cell responses mainly through
341 cytotoxic T lymphocyte (CTL) cross-reactions ^{39 - 43, 19}. B cells activation and NP
342 antibody response could also play a role in the protection against multiple influenza A
343 strains ^{44, 19}.
344 Since the pioneering studies of Wraith and his colleagues in the 1980s, it has been
345 shown that the influenza NP purified from virions can induce good but incomplete
346 protection against homologous and heterologous viral challenges in mice ⁴⁵. The same
347 group had previously shown that a combination of NP and HA primed the
348 development of cytotoxic T cells that were cross-reactive against other influenza A
349 viruses ⁴⁶.

350 Furthermore, several research groups reported the capacity of different NP-based
351 vaccines to accelerate viral clearance and prevent mortality in mice subsequently
352 challenged with either matched or mis-matched influenza A virus strains ⁴⁷. In that
353 regard, multiple studies on NP-based vaccines in the form of recombinant proteins ⁴⁷⁻
354 ⁵¹, DNA vaccines ^{40-42, 45-46} or viral vectors ^{52-56, 13}, confirmed the protective role of
355 CTL immune responses targeting NP.

356 In this context, we developed a more immunogenic version of NP, capable of eliciting
357 stronger and protective cytotoxic T cell responses at systemic and mucosal site (lung).
358 We initially fused NP to our first-generation oligomerization domain IMX313 ²¹, and
359 then to its improved version OVX313. Comparing to the IMX313, the C-terminal part
360 of the OVX313, includes a short arginine cationic sequence to facilitate vaccine
361 purification through chromatography. Through a DNA-based vaccine study, we
362 compared the level of immunogenicity generated by the “naked” NP antigen and of
363 NP fused to our technology, and found that the fusion to IMX313 increased the
364 immunogenicity of the NP antigen, which was further significantly boosted by fusion
365 to OVX313. Leveraging these DNA vaccine results, we developed a recombinant
366 protein vaccine harboring the NP protein from the prototype influenza H1N1
367 A/WSN/33 human strain ²⁷ fused to OVX313. Our technology platform (OVX313)
368 enables the production of an heptamerized form of the NP antigen, an OVX836
369 recombinant protein vaccine candidate. Several studies demonstrated that
370 oligomerization of antigens using this platform (through OVX313 tag or its previous
371 version IMX313) improves humoral and cellular immunogenicity in animals (Malaria
372 antigens ²³, Tuberculosis antigen 85A ²⁴, Malaria Pfs25 antigen ²⁵, HPV L2 antigen
373 ²⁶).

374 OVX836 enhanced antigen-uptake by DCs, and induced more NP-specific CD4+ and

375 CD8⁺ T cells responses as well as NP-specific IgG compared to NP alone. When we
376 compared OVX836 alone, in combination with an inactivated influenza vaccines
377 (IIV) and IIV alone in a mice immunization study, we showed that OVX836
378 generated significantly higher T cell responses than IIV alone. The cellular response
379 obtained with the combination of IIV and OVX836 was similar to the one obtained
380 with OVX836 alone. OVX836 induced high frequencies of cross-reactive NP specific
381 T cells, notably CD8⁺ T cells that were located in the lung parenchyma. For a long
382 time, intramuscular immunization has been considered as unable to generate mucosal
383 immune responses. In the 2016, Su, *et al.*, have published a large review of the
384 literature demonstrating that i.m. vaccination can promote both systemic and mucosal
385 immune responses, and protect against mucosal pathogen challenge ⁵⁷. In our study,
386 we demonstrated that i.m. immunization with OVX836 enhances NP-specific T cell
387 response localized in the lung parenchyma as shown by pentamer analysis using
388 CD45 intravascular staining method.

389 Mice challenge studies showed that OVX836 alone provides effective protection
390 against lethal infection with different subtypes of influenza A (A/WSN/33, H1N1 or
391 H3N2). Based on current scientific knowledge, we hypothesize that the protection
392 provided by administration of OVX836 alone is mostly mediated by CD8⁺ T cell
393 responses against the widely conserved NP antigen, with a potentiating action of
394 CD4⁺ T cell responses, and the potential contribution of NP-specific antibody
395 responses. In the 1980s, Wraith et al. made the hypothesis that immune responses
396 against NP would not prevent infection, but rather accelerate clearance of infected
397 cells ^{40,41}.

398 This hypothesis is in line with the weight curves observed for most of the groups
399 immunized with OVX836 alone, which experience initial body weight loss following

400 the viral challenge comparable to that of the PBS-vaccinated mice, but with less
401 severity and significantly faster recovery time. As a result, it shows the potential of
402 OVX836 to provide broad-spectrum protection against moderate to severe cases
403 caused by multiple A-strain viruses. Another attractive immunization strategy could
404 be to leverage two lines of attack by associating NP-targeted CD8⁺ T cell response
405 provided by OVX836 with the humoral response against HA generated by the IIV.
406 This is supported by our results showing decrease viral loads in mice, when OVX836
407 and IIV are used in combination, the IIV being either homologous or heterologous to
408 the viral strain used for the challenge. Such combined vaccination strategy could
409 prevent moderate to several cases cause by all influenza A subtypes and at the same
410 time limit viral dissemination even when antibodies are less effective in neutralizing
411 the virus.

412 Furthermore, we also noticed, with some viral strains used for the HAI assay, slightly
413 better pre-challenge HAI titers in the OVX836/IIV vaccinated groups compared to
414 those vaccinated with IIV alone. Although the real implications of this relatively mild
415 increase remain to be determined, a potential contribution of OVX836 to the HA-
416 specific humoral response induced by IIV might be worth further study.

417 The broad annual vaccination recommendation for people aged >6 months with
418 current IIV might represent a double-edged weapon in the long term, since on one
419 side, it confers partial yet important protection against circulating strains, but on the
420 other side, it could create a potential risk that vaccine recipients will not develop the
421 lasting heterosubtypic immunity usually acquired through repeated natural infections
422 ³⁸. An immunization strategy with a vaccine generating cellular response against NP,
423 like OVX836, could present a double advantage by protecting against moderate to
424 severe cases while not preventing subject's natural infection and the development of

425 lasting heterosubtypic immunity. Based on the mice experiments conducted so far,
426 OVX836 is a good candidate vaccine in order to effectively induce cell-mediated
427 immunity to a conserved antigen and provide wide protection against multiple A-
428 strain influenza viruses. As NP is highly conserved with >95% homology within A-
429 strains ⁵⁸, OVX836 represents an attractive standalone vaccination strategy to prevent
430 influenza caused by circulating or emerging A-strains, especially in a case of a
431 pandemic outbreak. In the context of limited effectiveness of licensed seasonal
432 vaccines, OVX836 immunization strategy could also be combined with current or
433 next generation vaccines triggering antibody responses against surface antigens in
434 order to provide complete protection against multiple circulating and emerging A and
435 B influenza subtypes.
436

437 **METHODS**

438 **OVX313, an improved version of the heptamerization domain IMX313**

439 IMX313 is a 55 amino acid protein domain, obtained as a hybrid of the C-terminal
440 fragments of two avian C4bp proteins that naturally oligomerize into heptamers. Of
441 note, in our IMX313 construction, avian C4bp sequences were engineered in order to
442 minimize identities with their human counterparts. We have previously observed that
443 following its fusion to a monomeric antigen, IMX313 allows the presentation of seven
444 copies of the antigen to the immune system, increasing both B & T cell
445 immunogenicity ^{21, 60}. Based on this observation, we designed for the present study
446 OVX313 as an optimized version of IMX313. We improved its oligomerization
447 domain by substituting the last seven amino acids of IMX313 (originally LQGLSKE)
448 by seven different amino acids (GRRRRRS), leaving the total length at 55 amino
449 acids which retains a heptameric core structure stabilized by monovalent
450 intermolecular interactions and disulfide bonds.

451 **Construction of plasmids**

452 Four plasmids were constructed for DNA immunizations by modifying pGT-h-NP, a
453 plasmid expressing NP ⁵⁹, to create four new ones. First, the human tPA signal
454 peptide sequence was amplified and inserted in-frame with the N-terminus of the NP
455 coding sequence in pGT-h-NP creating a eukaryotic expression plasmid (pIMX714)
456 for secreted NP ⁶⁰, using the forward primer IMX1305 (5'
457 CACTGAGTGACATCAAATCATGGATGCAATGAAGAGAGGGC 3') and the
458 reverse primer IMX1306 (5'
459 CGTAAGACCGTTTGGTGCCTTGGCTAGCTCTTCTGAATCGGGCATGGATTT
460 CC 3'). This plasmid was then modified by inserting PCR products encoding either
461 IMX313 creating the plasmid pIMX866 using the forward primer IMX1332 (5'

462 CTGATGTGTGCGGAGAGG 3') and the reverse primer IMX1039 (5'
463 TAGAAGGCACAGTCGAGG 3'), or OVX313 creating the plasmid pIMX867 using
464 the forward primer IMX1293 (5' GGTCAGGATGATCAAACGTGGG 3') and the
465 reverse primer IMX1039 (5' TAGAAGGCACAGTCGAGG 3'), from plasmids in
466 laboratory stocks. Finally, an empty vector was constructed (pIMX719), by digestion
467 of pGT-h-NP with the enzymes XbaI and HindIII, filling-in and re-ligating the ends,
468 thus deleting the sequence encoding tPA and NP. All plasmids were verified by DNA
469 sequencing. Two bacterial expression plasmids were used for overproducing the
470 nucleoprotein of the prototype human influenza isolate WS ²⁷. A synthetic gene,
471 codon optimized for expression in *Escherichia coli* and encoding OVX836, was
472 purchased from DNA2.0 (now ATUMBIO) in the bacterial expression vector
473 pDR441-SR. This plasmid, called pOVX836, was modified to delete the sequence
474 encoding OVX313, creating the expression plasmid for NP (strain A/Wilson-
475 Smith/1933) called pNP.

476 **Protein production and characterization**

477 Both the NP and the OVX836 proteins were produced by using the bacterial strain
478 BL21. Briefly, when bacterial growth reached the logarithmic phase, 0.5mM
479 isopropyl β -D-1-thiogalactopyranoside (IPTG) was used to induce expression for 16
480 hours at 25°C. Recombinant NP and OVX836 proteins were purified by means of
481 affinity chromatography on a Heparin-Sepharose column. The mutated form of NP
482 (mut-NP and mut-OVX836) was an E339A/R416A mutant NP that did not self-
483 associate and formed only monomers ^{29, 60} (Supplementary Figure 1b).

484 **SDS-PAGE analysis**

485 Protein purity was evaluated by SDS-PAGE. Recombinant proteins were analyzed
486 using a Criterion 4-12% Bis-tris gel (Bio-Rad), following the suppliers' instructions.

487 Briefly, proteins were denatured and reduced using NuPAGE LDS sample buffer (4X,
488 Thermo) and NuPAGE reducing agent (10X, Thermo), respectively. The samples
489 were heated at 70°C for 10 minutes and then 0.5 µg of proteins were loaded on the
490 Criterion 4-12% Bis-tris gel. The electrophoretic separation was carried out using a
491 MOPS SDS running buffer at a constant voltage (200 V) for 50 minutes, using the
492 NuPAGE antioxidant (Life Technologies). Proteins were detected using Instant Blue
493 (Euromedex). Precision Plus Protein Standards (Bio-Rad) were used as molecular
494 weight marker. Gel images were acquired with a Chemidoc Touch Imager (Bio-Rad)
495 and images were then analyzed using the Image-Lab software (Bio-Rad).

496 **Western-blot**

497 Western blotting, using an antibody directed against Nucleoprotein antigen (MAb
498 anti-NP, Clone 7B4G10G8), was performed to establish the identities of OVX836 and
499 NP. Recombinant proteins were denatured and reduced as described previously. 1D-
500 PAGE was performed as described above. The gel was then blotted onto a
501 nitrocellulose membrane using a Trans-blot transfer system and a Trans-blot
502 nitrocellulose pack (Bio-Rad) following the suppliers' instructions. Precision Plus
503 Protein Western C Standards (Bio-Rad) were used as molecular weight markers. The
504 membrane was blocked with 3% BSA in TBS-T (0.1%T, w/v) and then probed with
505 the monoclonal antibody directed against NP antigen (MAb anti-NP, 1/3000).
506 Peroxidase-conjugated goat anti-mouse secondary antibodies were purchased from
507 Thermo (1/30000), and the chemiluminescence detection kit (Clarity ECL Western-
508 blot substrate) from Bio-Rad. Western blot signals were acquired with an instrument
509 for western blot imaging (Chemidoc Touch Imager, Bio-Rad) and images were
510 analyzed using the Image-Lab software (Bio-Rad).

511 **Liquid chromatography**

512 Reversed-phase ultra-high-performance liquid chromatography (RP-UHPLC) was
513 performed to provide information on protein homogeneity. Protein species were
514 separated on a non-polar stationary phase with a gradient of hydrophobicity. Briefly,
515 around 4.5 µg of protein were injected on a MAbPac RP column (Thermo, 4 µm, 2.1
516 x 100 mm), thermostated at 40°C. The elution (0.4 mL/min) was performed using a
517 linear gradient of acetonitrile with 0.1% TFA and monitored by UV detection at 280
518 nm. The reducing agent TCEP (10mM final) was added to reduce proteins for RP-
519 UHPCUV analysis.

520 High performance size exclusion chromatography (SE-UHPLC-UV) was performed
521 in order to analyze product quaternary structures and sizes of recombinant molecules.
522 Briefly, a BEH SEC450 (Waters, 4.6x150 mm, guard 4.6x30 mm), equilibrated at
523 30°C, was used in combination with 50 mM Na/K₂ phosphate buffer pH 6.8, 0.5M
524 NaCl, 0.8M arginine mobile phase. Ten µg of protein sample was loaded onto the
525 column and monitored by UV detection at 280 nm.

526 **Cell preparation and flow cytometry analysis**

527 Flow cytometry and fluorescence-based imaging flow cytometry were used to analyze
528 the uptake of proteins labelled with Alexa Fluor 647 fluorochrome (Molecular Probes,
529 Invitrogen Life Technologies). Bone marrow DC were generated from C57BL/6
530 mice, cells were isolated by flushing femurs and tibiae with culture medium RPMI
531 1640 (Invitrogen) containing 10% heat-inactivated FBS, 10 µg/ml gentamicin, 2 mM
532 l-glutamine (Invitrogen), 50 µM 2-ME (Sigma-Aldrich), and 10 mM HEPES
533 (Invitrogen). Cells were filtrated through a 100-µm nylon cell strainer (BD Falcon),
534 centrifuged, and cultured at 2×10^6 cells/ml in medium supplemented with 100 ng/ml
535 recombinant human Flt3l (Amgen)⁶¹. Flt3L-DC at day 7 to 8 of culture were
536 incubated for the indicated time with Alexa647-labelled proteins. DC were washed

537 and incubated 15 minutes with Live Dead reagent in PBS 1X (Life technologies, 0.5
538 μ l per well). DCs were collected and stained 45 min at 4°C with antibodies specific
539 for the murine cell surface antigens in FACS buffer (PBS1X, 1% FCS, 0.09% azide):
540 CD11c (clone HL3), B220 (RA3-6B2), CD24 (M1/69), CD172a (P84) (all BD
541 Biosciences). Prior to acquisition, cells were fixed in 2% paraformaldehyde.
542 Acquisition was performed on LSR Fortessa flow cytometer (BD Biosciences) and
543 post-collection data were analyzed using FlowJo software (TreeStar). For fluorescent
544 imaging experiments, data and images were collected on an Amnis ImageStreamX
545 Mark II, and at least 5000 events per sample were acquired. Data were analyzed using
546 IDEAS software (EMD Millipore) and magnification of $\times 60$ was used for all images
547 shown.

548 For in vivo experiments, intravascular staining with anti-CD45-BV421 was
549 performed 3 min before killing of the mice, thereby staining all intravascular, but not
550 parenchymal, lymphocytes in order to identify lung resident memory CD8⁺ T cells as
551 described⁶². Mouse spleens and lungs were collected and dissociated by mechanical
552 disruption with GentleMACS Dissociator (spleens or lungs) (Miltenyi Biotec). Single
553 cell suspensions were stained with antibodies against CD45 (30F11), CD8a (53.6),
554 CD44 (IM7) (all BD Biosciences), and analyzed by flow cytometry. NP-specific CD8⁺
555 T cells in lungs and spleens were detected using pentamer staining, cells were
556 incubated with MHC class I (H-2Db) pentamers specific for influenza virus NP₃₆₆₋₃₇₄
557 - PE (H2Db-ASNENMETM) (Proimmune).

558 **Animals and immunizations**

559 Six-week-old female C57BL/6 mice (Charles River Laboratories, Lyon, France) were
560 used in all experiments, except for the challenge test, for which six-week-old female
561 Balb/c mice (Charles River Laboratories, Quebec, Canada) were also used as

562 specified on each case. Animals were maintained under specific pathogen-free
563 conditions, with *ad libitum* access to food and water. All animal procedures were
564 approved by the Institutional Animal Care ethics committee of the Plateau de
565 Biologie Expérimental de la Souris (CECCAPP_ENS_2015_004) and the Institutional
566 Animal Care Committee of the Centre Hospitalier Universitaire de Québec (CPAC
567 #2013-134), in full accordance with European regulations and the guidelines of the
568 Canadian Council on Animal Care, respectively.

569 DNA immunizations: To determine the effects of IMX313 and OVX313 on the
570 immunogenicity of NP in plasmids, DNAs (50 µg) in endotoxin-free PBS were
571 administered intramuscularly (i.m.) into the gastrocnemius muscle on days 0 and 14.

572 Immunogenicity and dose-response with recombinant proteins: to compare the
573 immunogenicity of the OVX836 protein to that of NP, mice were i.m. immunized
574 twice three weeks apart with 6.25, 12.5, 25 or 50 µg of the respective proteins.

575 Co-administration of OVX836 and the seasonal influenza vaccine: to determine
576 whether the co-administration of OVX836 protein with IIV vaccines could potentially
577 stimulate both highly specific T cell responses to nucleoprotein and increased
578 antibody responses to hemagglutinins present in the seasonal vaccine, groups of
579 female C57BL/6 mice were i.m. immunized twice, 21 days apart with 25 µg of
580 OVX836, with or without IIV (Fluarix®, GSK 2014-2015 formulation, 1.5 µg of each
581 HA in 50 µL). Immunizations were performed by injection into the gastrocnemius
582 muscle or two different muscles (gastrocnemius and calf muscle) for double
583 administration (OVX836 and IIV), with both injections being administered in the
584 same hind limb.

585 Blood samples were collected before each immunization and two weeks or (14 days)
586 after the booster immunization, mice were sacrificed and both serum, lung and
587 splenocytes were collected from each mouse and processed individually.

588 **Mice Challenge studies**

589 Influenza challenge studies were conducted at the Infectious Disease Research Centre
590 of the CHU de Quebec and Laval University (Quebec, Canada) and at the Plateau de
591 Biologie Expérimental de la Souris (Lyon, France). Groups of 12 female C57BL/6 or
592 Balb/c mice were immunized in two different muscles of the same hind limb with
593 either 25 µg (in 25 µl) of the OVX836 protein, of the IIV (Fluviral®, GSK 2014-2015
594 or Influvac®, 1.5 µg of each HA in 50 µL), or a combination of both (OVX836/IIV).
595 Two immunizations (21 days apart) were performed for each group, and a PBS-
596 immunized group was included as control. Three weeks after the second
597 immunization, mice were again lightly anesthetized by isoflurane and infected
598 through an intranasal instillation with 20 µl of a viral suspension, containing either
599 3LD50 of influenza H1N1 A/California/7/2009, 1LD50 (in Balb/c mice) or 2LD50 (in
600 C57BL/6 mice) of H1N1 A/WSN/33 or 1LD50 of H3N2 A/Victoria/5/72 strain, as
601 indicated in each case. Of note, the H1N1 A/California/7/2009 strain is homologous
602 to the H1N1 strain present in the IIV vaccine, while the other two strains used in this
603 study are not. All mice were monitored daily for survival and weight loss during 14
604 days. Four mice per group were randomly chosen and sacrificed on day 4
605 (A/California/7/2009 and A/WSN/33) or 5 (A/Victoria/5/72) post-challenge, and their
606 lungs were aseptically removed for the determination of lung viral titers (LVTs) by
607 standard plaque assay in MDCK cells. Serum samples were collected from each
608 animal before each immunization and viral challenge, as well as on day 21 post-
609 challenge, to evaluate the specific IgG antibody response by Hemagglutination

610 Inhibition (HAI) assays. Serologic tests were performed against the same virus strains
611 used for the viral challenge as well as the strains contained in the IIV vaccine
612 (A/California/7/2009 (H1N1), A/Texas/50/2012 and B/Massachusetts/2/2012),
613 following the standard WHO guidelines ⁶³.

614 **Ex-vivo IFN γ Enzyme-Linked ImmunoSpot (ELISpot) Assay**

615 Influenza NP-specific T cells secreting IFN- γ were enumerated using an IFN- γ
616 ELISPOT assay (Mabtech). Lymphocytes were isolated from the spleen and the lung
617 from individual mice. CD4⁺ T cells were purified by positive selection using a
618 MACS Isolation Kit for mice with CD4 (L3T4). ELISpot plates were coated with the
619 capture mAb (#3321-2H) then incubated overnight at 4°C according to the
620 instruction manual of Mabtech. Then 2×10^5 T cells and CD4⁺ cells were cultured for
621 20 hours at 37°C/5% CO₂ with 5 μ g/mL of recombinant NP protein (OSIVAX). In
622 addition, 2×10^5 T cells were stimulated in similar conditions with 5 μ g/mL of the
623 NP₃₆₆₋₃₇₄ (GenScript) immunodominant peptide epitope in C57BL/6 mice presented
624 by H-2Db to stimulate CD8⁺ T cells ⁶⁴. Concavalin A (Sigma) was used as a positive
625 control and unstimulated splenocytes/lung cells were used as negative controls. Spots
626 were counted with an ELISPOT reader system (CTL-ImmunoSpot® S6 Ultra-V). The
627 number of protein-or peptide reactive cells was represented as spot forming cells
628 (SFCs) per 2×10^5 cells per well.

629 **Cytokine production by ELISA after in vitro stimulation**

630 For analysis of cytokine secretion, lung cells were cultured (2×10^6 cells/ml) for 48h
631 with 5 μ g/ml peptide NP₃₆₆₋₃₇₄. Supernatants were collected and cytokine production
632 was measured by sandwich ELISA kit (Mabtech) for IFN γ (3511-1H), IL-2 (3311-
633 1H), TNF α (3441-1H), and IL-4 (3321-1H) according to the protocols provided by the

634 manufacturer. Cytokine concentrations in pg/ml were quantified using an appropriate
635 standard curve.

636 **Antibody Enzyme Linked Immunosorbent Assay (ELISA)**

637 Levels of Immunoglobulin G (IgG) were measured in serum samples collected on
638 days 35. The 96-well ELISA plates were pre-coated with 100 μ L of recombinant NP
639 (OSIVAX) at 5 μ g/mL overnight at 4 °C. A total of 100 μ l of serial 2-fold dilutions of
640 sera from each group of immunized mice were added to each well and incubated for 2
641 h at 25 °C. Bound antibody was detected with goat anti-mouse IgG-HRP (Life
642 Technology) and finally, 100 μ l of tetramethylbenzidine (TMB) (Interchim) substrate
643 was added to each well. The antibody levels in serum were expressed as logarithm of
644 endpoint dilution titer and this is defined as the reciprocal of the highest analytic
645 dilution that gives a reading 3-fold over the mean O.D. 650 value of the negative-
646 control mice serum at the 1/100 dilution. A value of 1.70 (log) was arbitrarily chosen
647 to illustrate a titer <100.

648 **Statistical Analysis**

649 Statistical analysis. The plotting of data and statistical analysis were performed using
650 GraphPad Prism 7 software. Statistical significance was determined using the
651 unpaired, one-way analysis of variance (ANOVA) with Tukey's multiple
652 comparisons test or non-parametric Kruskal-Wallis test followed by Dunn's multiple
653 comparisons test. Differences were considered significant if the p value was $p < 0.05$.
654 Survival rates of mice were compared using Kaplan Meier survival analysis, and
655 statistical significance was assessed using the Log-Rank (Mantel-Cox) test. For
656 weight loss curves, groups were compared at each day post-infection using Kruskal-
657 Wallis test followed by Dunn's multiple comparison test.

658

659 **Data availability**

660 The data that support the findings of this study are available from the corresponding
661 author upon reasonable request.

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670

671 **Competing Interests**

672 J Del Campo and F Hill are named as inventors on a family of patents/patent
673 applications “Influenza Nucleoprotein vaccines” (WO 2014/090905, WO
674 2014/147087), and both are shareholders and employees of OSIVAX. All other
675 authors declare no competing interests.

676

677 **Author Contributions**

678 JDC, FH, FN, ALV conceived and designed the study. JDC, FH designed the
679 molecular structures. JPV, and MH conducted protein production and purification. JB
680 conducted analytical characterization. JDC, SD planned and conducted the *in vivo*
681 immunologic experiments. JDC, GB, MEH conducted the challenge studies. MRC,
682 AP conducted the virology work and contributed to challenge studies coordination,
683 virologic tests, data analysis. SD, YL, JM conceived and designed the study on DC,
684 CD8+ T cells, data acquisition, analysis and interpretation of the results. JDC and FH
685 wrote the manuscript. SD, AP contributed to the manuscript in their respective
686 section. All authors contributed critically to the drafts and gave final approval for

687 publication.

688

689 **Footnotes**

690 §Andres Pizzorno and Sophia Djebali contributed equally to this work.

691

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878 **Figure Legends**

879 **Figure 1. DNA based immunization with NP fused to a small oligomerization**
880 **domain**

881 (a) Primary structure of OVX313 oligomerization domain and alignment with
882 IMX313 and the homologous human C4BP-alpha. Residues in bold type are identical.
883 Cysteine residues are highlighted in yellow. (b) C57BL/6 mice were immunized with
884 DNA plasmid encoding NP, NP-IMX313, OVX836 (NP-OVX313), or the empty
885 vector by the intramuscular route. NP-specific IFN- γ secreting cells were evaluated
886 by ELISpot in splenic cells. The results represent the mean (line) and individual data
887 points of each of the five mice with standard deviation of the mean (SD). (c) IFN- γ
888 secreting CD4⁺ and CD8⁺ T cell, in the splenocytes stimulated with 5 μ g/ml of NP
889 protein or 5 μ g/ml of NP₃₆₆₋₃₇₄, respectively. (d) Serum anti-NP IgG were determined
890 using ELISA. Sera were individually tested in serial dilutions against purified NP
891 protein. The mean (line) of each group is represented with SD. Levels of IgG are
892 expressed as Log (endpoint dilution titer). Differences were assessed by 1-way
893 ANOVA followed by Tukey's multiple comparison test with 95% confidence interval,
894 $p < 0.05$ is considered significant. The asterisks refer to the level of significance
895 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$). Results from a representative
896 experiment of two performed is shown.

897

898 **Figure 2. OVX836 protein is an oligomeric form of NP with improved**
899 **immunogenicity**

900 (a) Representative SDS-PAGE analysis of OVX836 and NP proteins. Polypeptides
901 were fractionated on 4-12 % Bis-tris gels and stained with Instant Blue, total protein
902 were stained (left panel) or blotted onto a membrane and probed with anti-NP Mab
903 (right panel). OVX836 and NP migrated at 64 and 56 kDa, respectively (lanes 1 and
904 2). Molecular weight (MW) markers (lanes M). The SDS-PAGE and the Western-blot
905 derive from the same experiment and were processed in the same day. (b)
906 Recombinant protein species were separated by reversed-phase (RP) liquid
907 chromatography: The R_t of ~ 5.45 min (red chromatogram) corresponds to the
908 oligomeric structure of OVX836, and the R_t of ~ 5.22 min to the monomer of NP.
909 Noncovalently bonded sub-units, most likely dimers, trimers and tetramers of NP,
910 were chromatographically separated from oligomeric OVX836 following reduction
911 (red dashed line). Intensity (Int). (c) Schematic representation of OVX836 protein
912 heptamer. (d) Kinetics of incorporation of OVX836-Alexa647 by Flt3L-DC Bone
913 marrow. DC were incubated for 2, 6 or 24 hours with OVX836-Alexa647 or NP-
914 Alexa647 (50 μ g/ml) at 37°C and surface stained for CD11c and B220. Alexa647 MFI
915 of cDC (CD11c+, B220-) and pDC (CD11c+, B220+) populations were measured.
916 Data show means \pm S.D. of two independent experiments. (e) Comparison of NP-
917 specific IFN- γ producing cells evaluated by ELISpot in total splenic cells (C57BL/6
918 mice immunized twice, intramuscularly, with 6.25, 12.5, 25 and 50 μ g of NP or
919 OVX836). Results show number of IFN- γ secreting cells per 2×10^5 cells per well. (f)
920 NP-specific IgG were measured by ELISA in serum from the same C57BL/6 mice
921 groups. Levels of IgG are expressed as Log (endpoint dilution titer). The results
922 represent the mean (line) and individual data points of each of the five mice with SD.

923 Differences were assessed by 1-way ANOVA followed by Tukey's Multiple
924 comparison test with 95% confidence intervals; $p < 0.05$ is considered significant
925 (**** $p < 0.0001$) or by two-way ANOVA followed by a Dunnett's multiple
926 comparison test (multiple time points) (** $p < 0.01$; **** $p < 0.0001$). These
927 experiments were performed 3 times with similar results.

928 **Figure 3. OVX836 vaccine alone or combined with inactivated seasonal**
929 **influenza vaccine induced humoral and cellular responses in the periphery**
930 **and in the lung**

931 C57BL/6 mice were immunized twice, intramuscularly with 25 μ g of OVX836; with
932 50 μ L of IIV (1.5 μ g of each HA); or with both combined (OVX836/IIV), by injection
933 into two different muscles in the same hind limb. A group injected with PBS was used
934 as control. (a, b) cellular immunogenicity following immunization in mice. NP-
935 specific IFN- γ secreting T cells (Spot forming cells SFC) evaluated by ELISPOT per
936 2×10^5 cells in the spleen and in the lung compartments. Results are shown for 3
937 independent experiments. The results represent the mean (line) and individual data
938 points of each of the five mice. (c, d) Comparison of specific IFN- γ secreting CD4+
939 and CD8+T-cell evaluated by ELISPOT in the spleen or in the lung compartments. (e,
940 f) NP-specific CD8+T cells in the spleen and lung compartments were detected by
941 flow cytometry using pentamer staining (H2Db-ASNENMETM). (e) Number of
942 Pentamer CD8+T cells in the spleen and (f) in the lung. Lung parenchyma (resident)
943 cells were identified using anti-CD45 intra-vascular staining. Data are representative
944 of three independent experiments (mean of five mice per group). Differences were
945 assessing by 1-way ANOVA followed by Tukey's Multiple comparison test with 95%
946 confidence intervals; $p < 0.05$ is considered significant (* $p < 0.05$, ** $p < 0.01$,
947 **** $p < 0.0001$).

948

949 **Figure 4. Vaccination with OVX836 protects mice against lethal infection with**
950 **influenza H1N1 A/WSN/33 strain**

951 Groups of 12 female C57BL/6 (a, c, e) or Balb/c mice (b, d e) received two 21-days-
952 apart i.m. immunizations with either PBS, IIV, OVX836, or a combination of IIV
953 and OVX836 (OVX836/IIV). Three weeks after the second immunization, mice were
954 i.n. infected with 2LD50 (C57BL/6) or 1LD50 (Balb/c) of H1N1 A/WSN/33 virus. (a-
955 b) Percent survival rates. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Log-Rank (Mantel-
956 Cox) or Kruskal-Wallis (Dunn's multiple comparison) tests. (c-d) mean weight
957 changes (\pm SEM), (e-f) mean lung viral titers expressed as log (PFU/mL \pm SD) on day
958 4 post infection. Individual virus titers are given and lines represent averages. 1.7 log
959 PFU/mL is the detection limit of the technique (dotted line). Statistical significance of
960 the variance between multiple groups was calculated with Kruskal-Wallis test
961 followed by Dunn's multiple comparison test (* $p < 0.05$, *** $p < 0.001$, **** p
962 < 0.0001).

963 **Figure 5. Vaccination with OVX836 induces broad protection against lethal**
964 **challenge with different influenza A subtypes**

965 Groups of 12 female Balb/c mice received two 21-days-apart i.m. immunizations
966 with either PBS, inactivated influenza vaccine (IIV), OVX836, or a combination of
967 IIV and OVX836 (OVX836/IIV). Three weeks after the second immunization, mice
968 were i.n. infected with either 3LD50 of influenza H1N1 A/California/7/2009 (left
969 panel) or 1LD50 of H3N2 A/Victoria/5/72 (right panel) virus. (a, b) Percent survival
970 rates. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Log-Rank (Mantel-Cox) (c-d) mean
971 weight changes (\pm SEM), (e, f) mean lung viral titers expressed as log (PFU/mL \pm SD)
972 on day 4 or 5 post infection. Individual virus titers are given and lines represent
973 averages. 1.7 log PFU/mL is the detection limit of the technique (dotted line).
974 Statistical significance of the variance between multiple groups was calculated with
975 Kruskal-Wallis test followed by Dunn's multiple comparison test (* $p < 0.05$, *** p
976 < 0.001 , **** $p < 0.0001$).

977 **Table 1. Hemagglutination Inhibition (HAI) titers. Serologic tests against the**
978 **virus strain used for the viral challenge as well as the strains contained in the IIV**
979 **vaccine**

980 HAI titers were measured in pooled serum samples (3 pools per group) and expressed
981 as the reciprocal of the limit dilution. Serologic tests were performed against the same
982 virus strain used for the viral challenge (H1N1 A/WSN/33 virus), as well as the
983 strains contained in the IIV vaccine (A/California/7/2009 (H1N1), A/Texas/50/2012
984 and B/Massachusetts/2/2012).

a

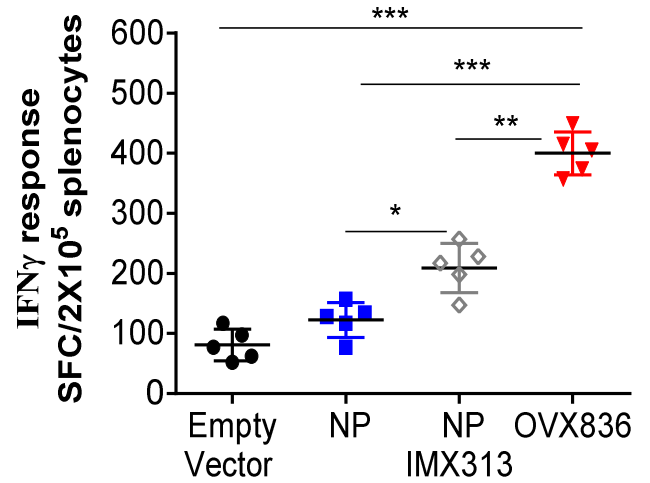
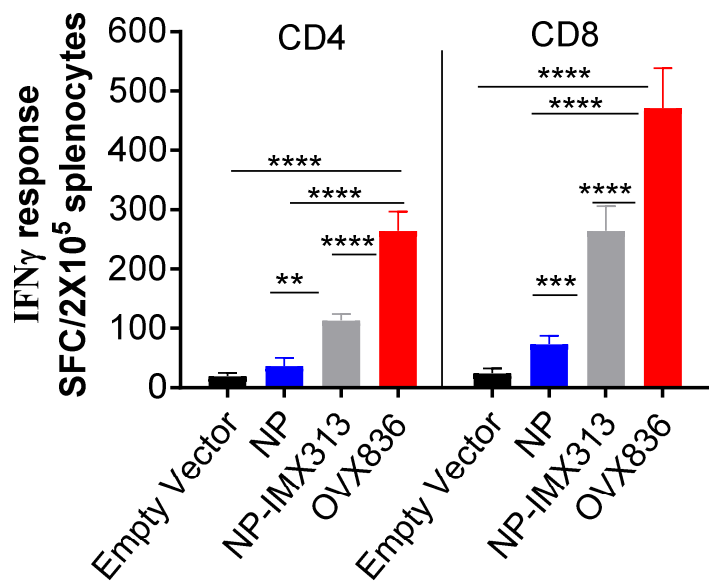
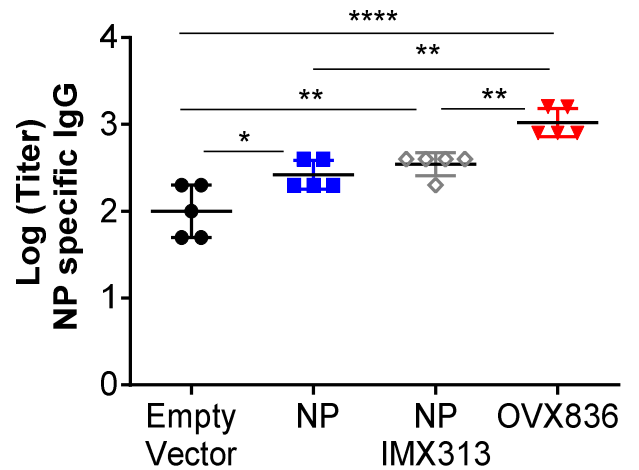
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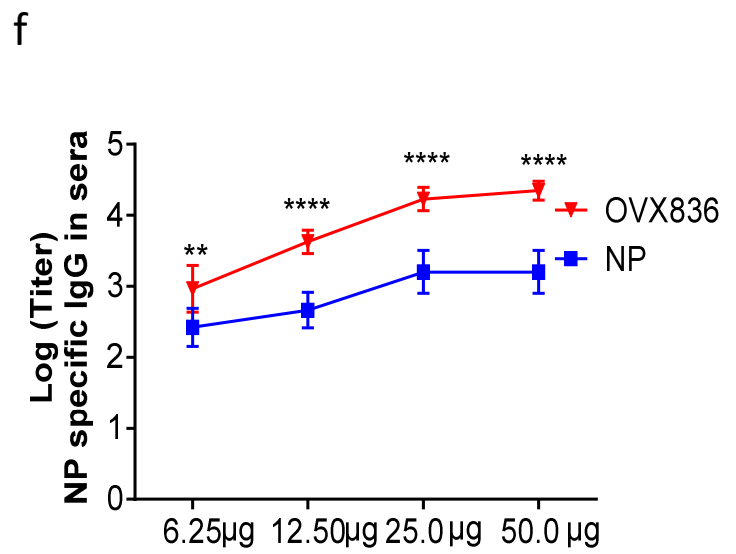
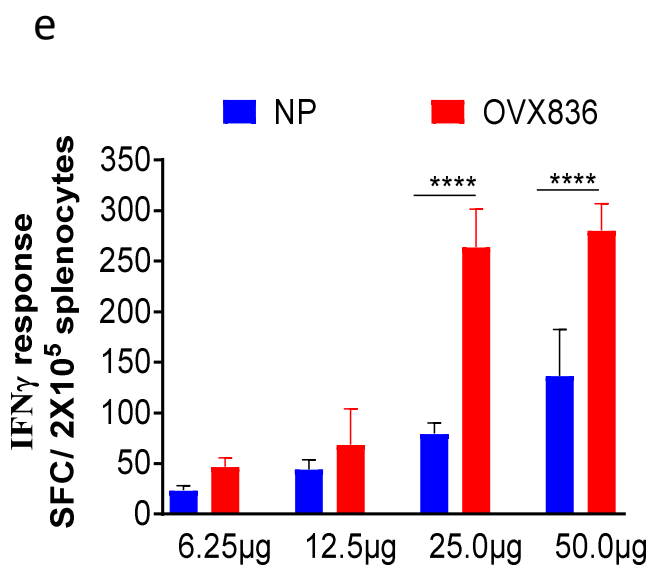
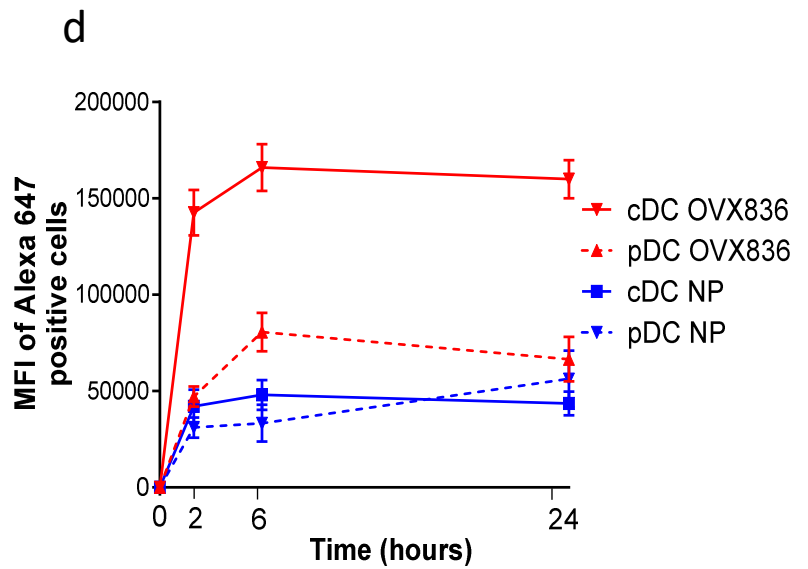
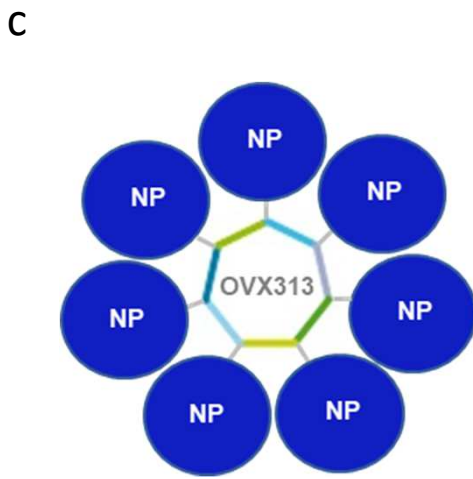
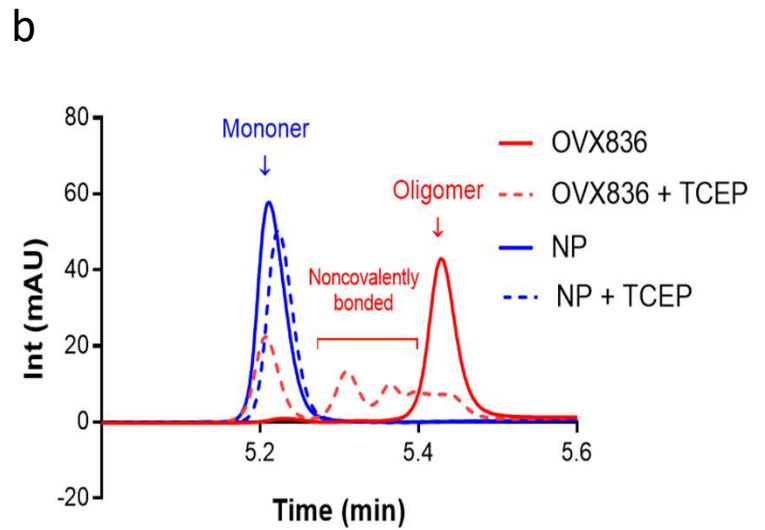
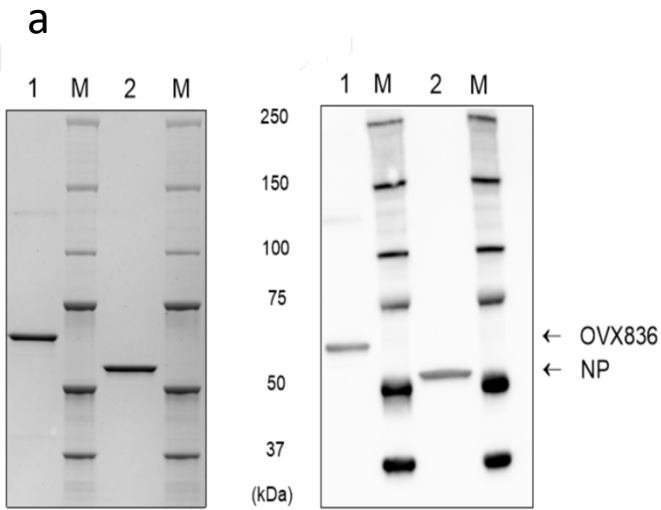
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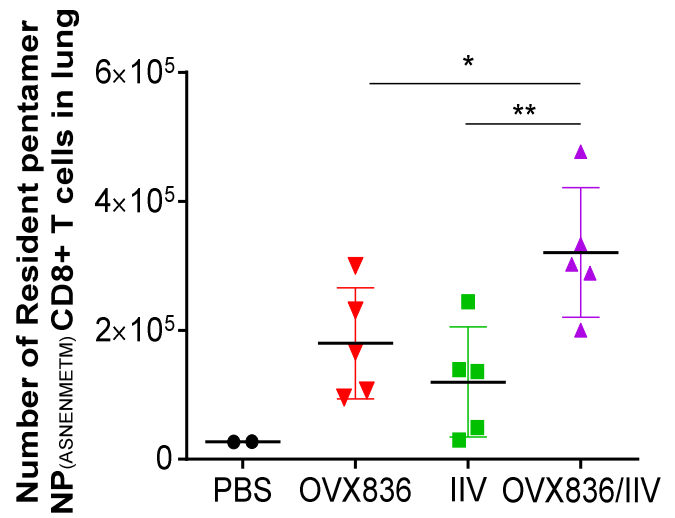
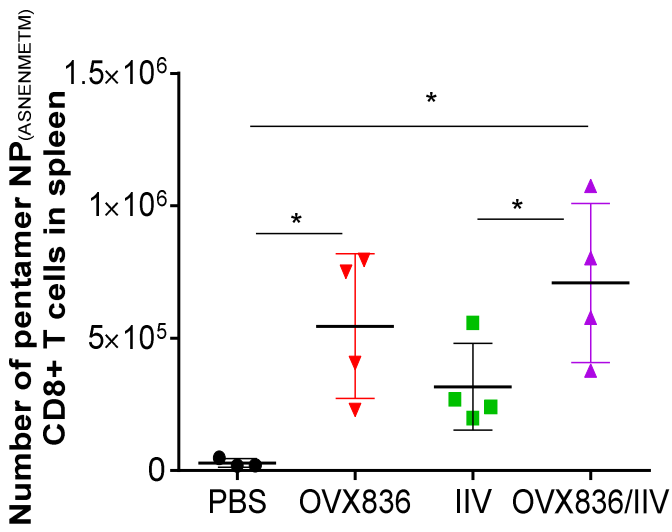
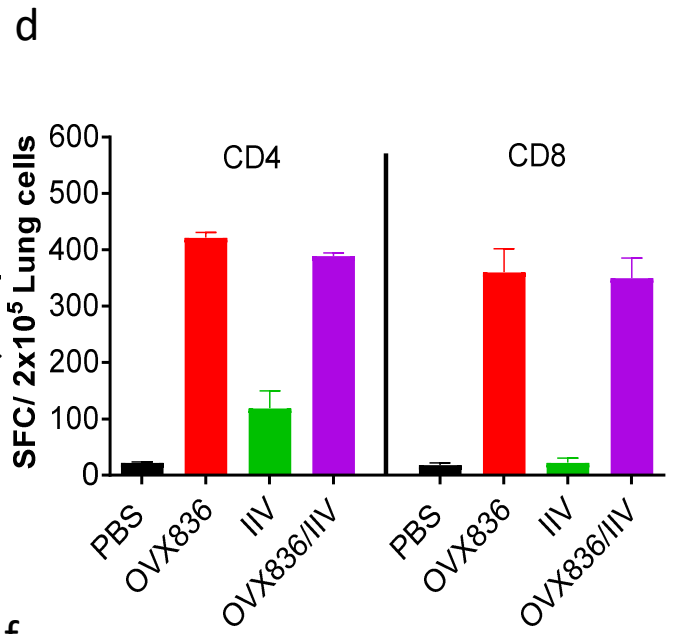
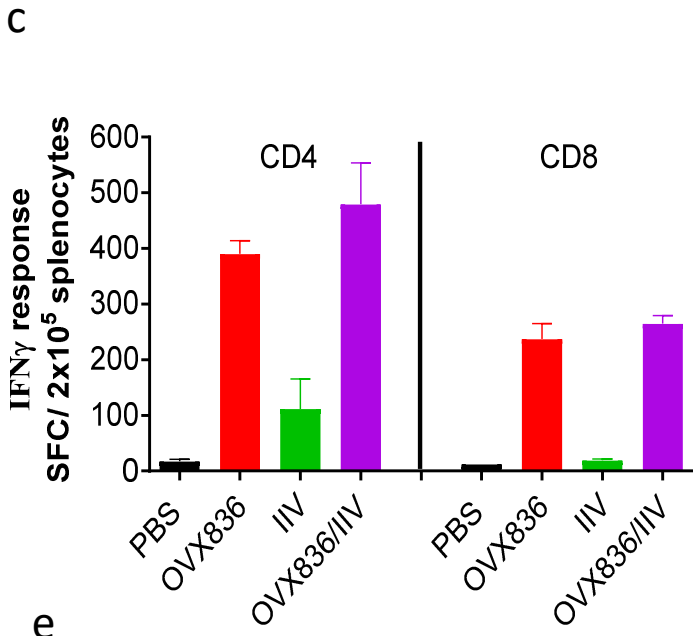
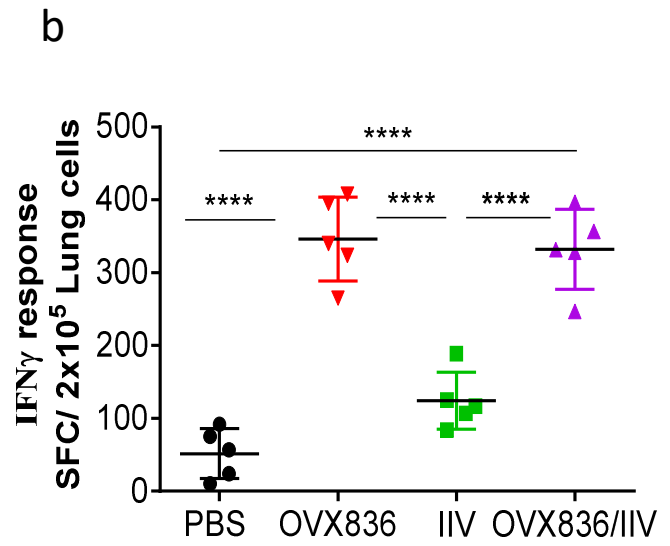
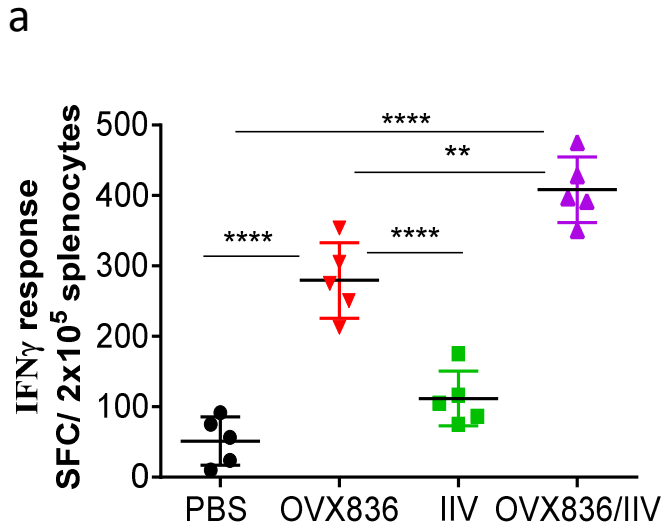
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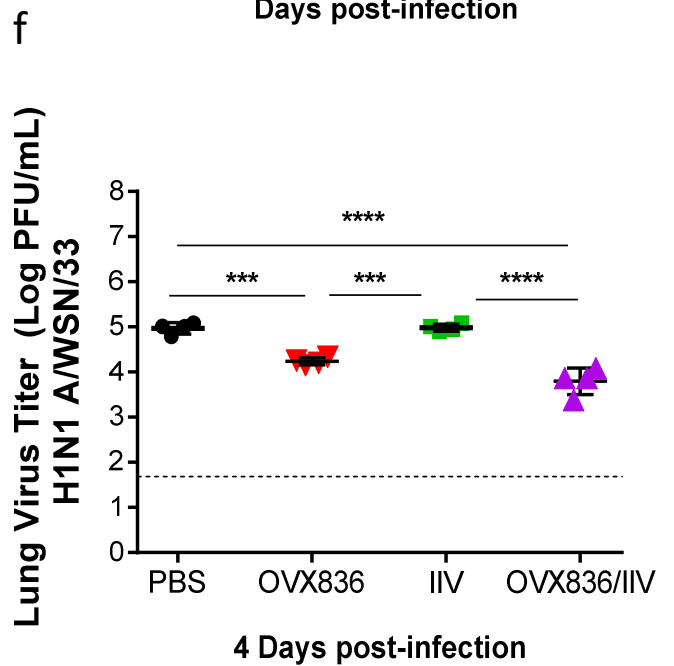
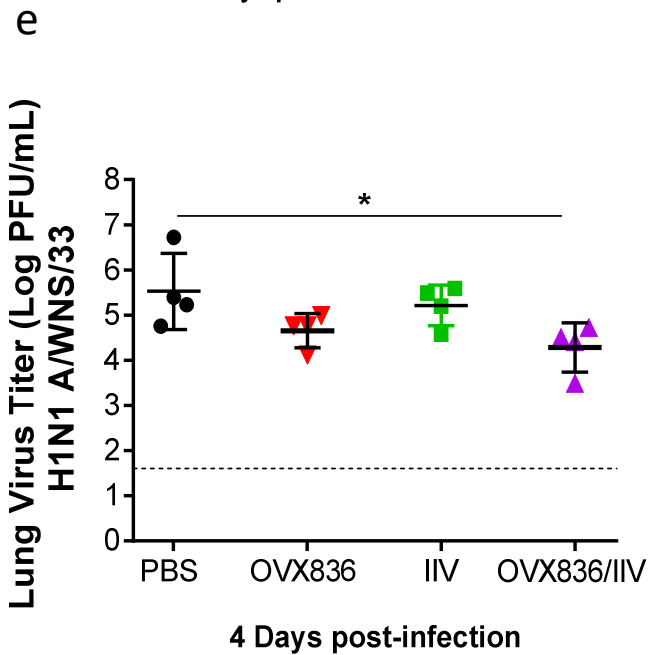
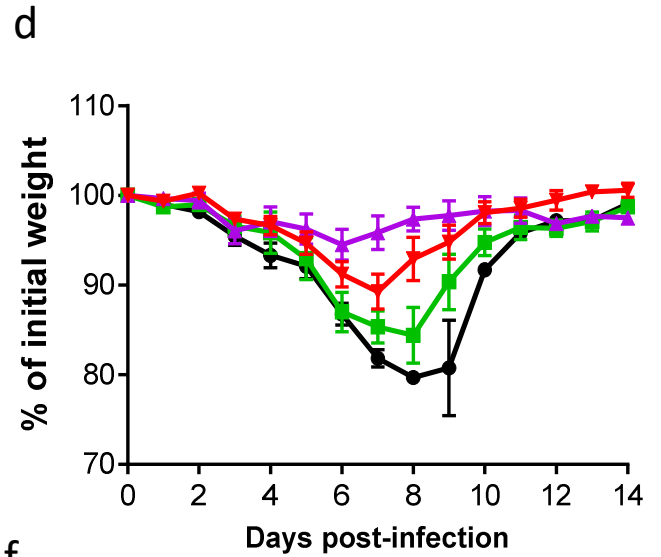
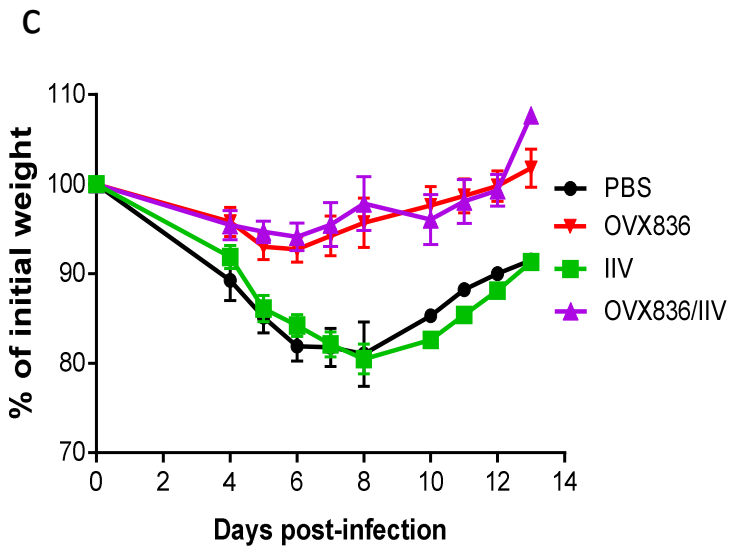
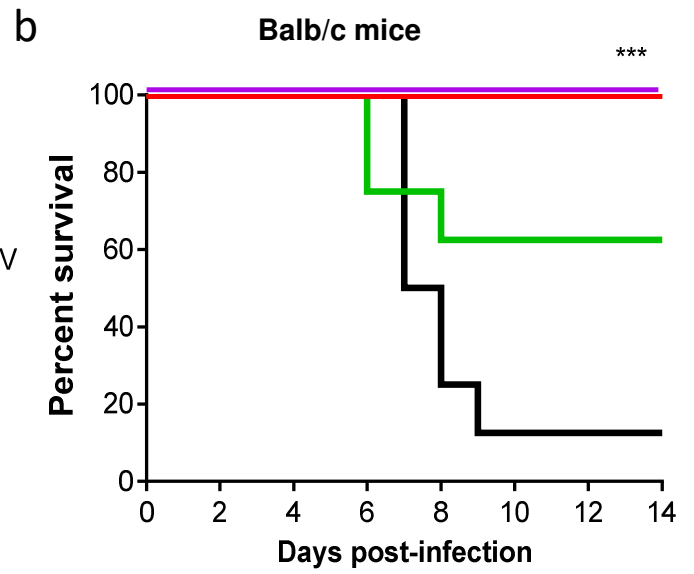
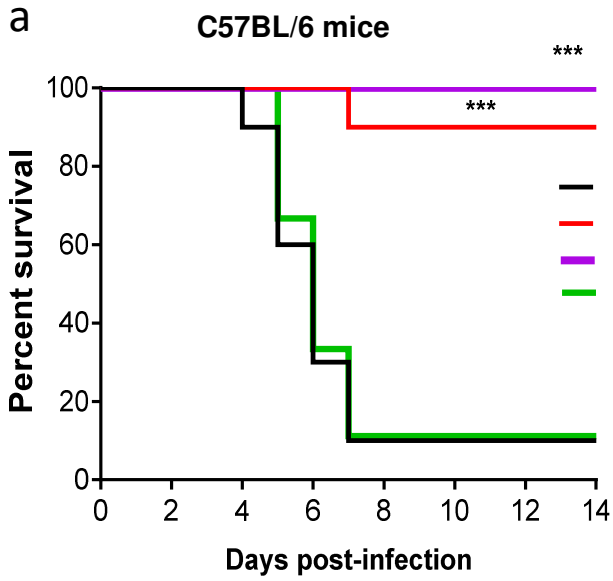
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b**c****d**







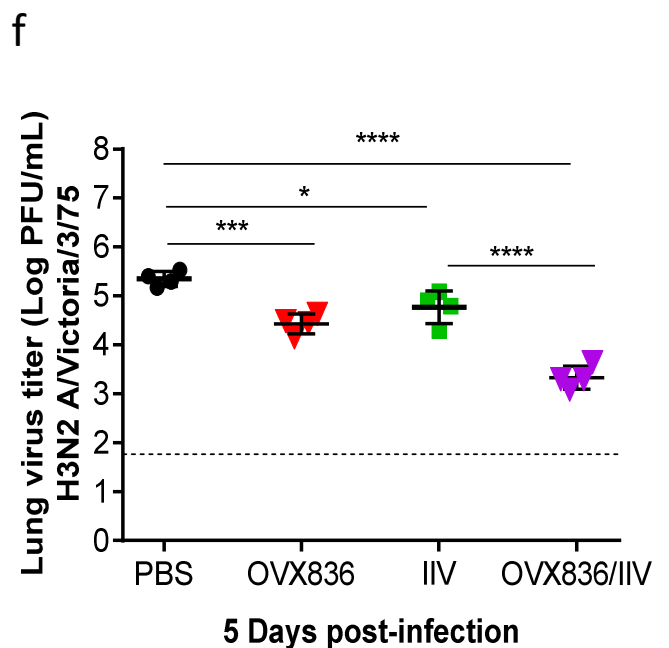
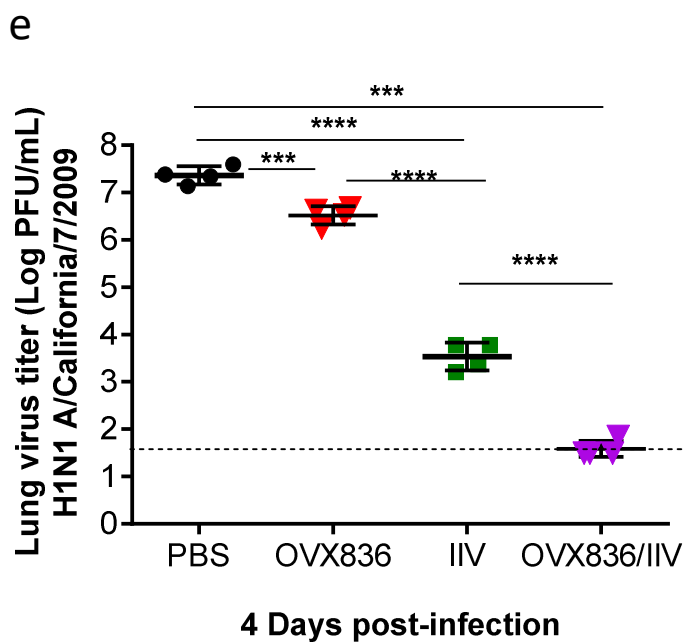
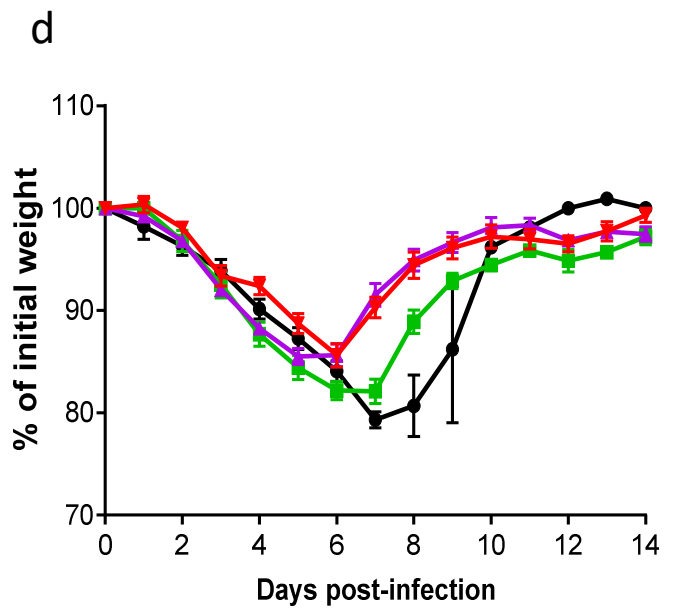
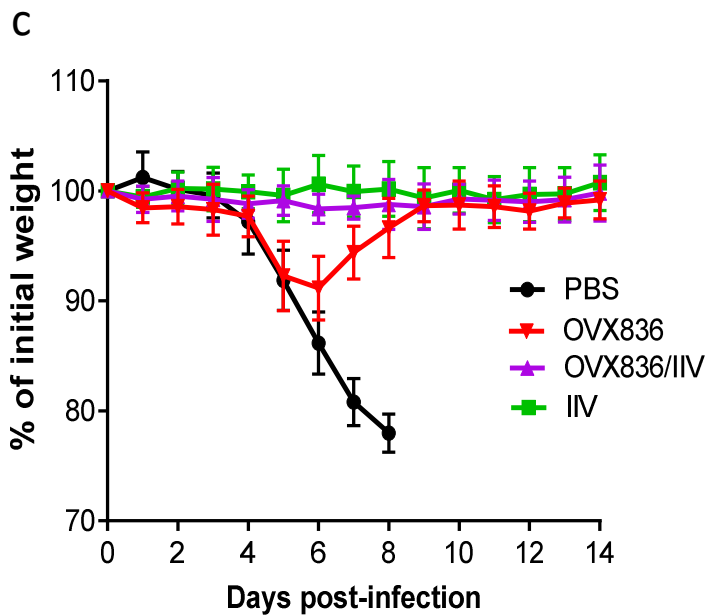
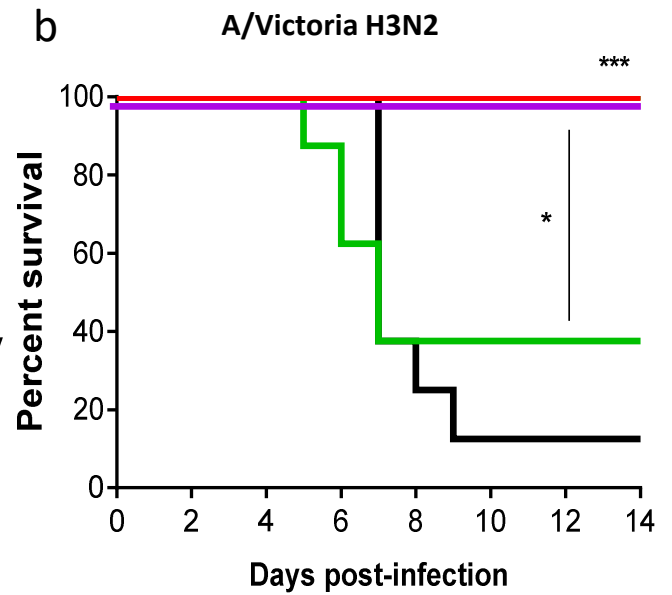
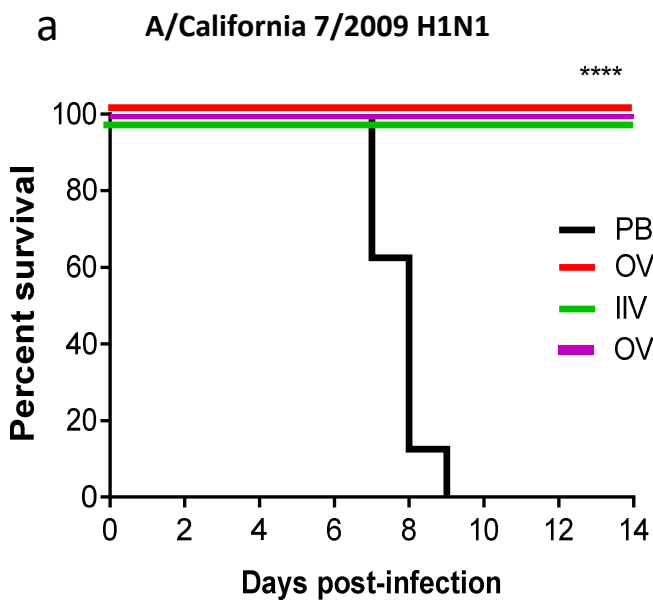


Table 1 Hemagglutination Inhibition (HAI) titers. Serologic tests against the virus strain used for the viral challenge as well as the strains contained in the IIV vaccine

		Viral strain used for HAI assay								
		A/WSN/33		A/California/2009		A/Texas/54		B/Massachusetts		
		Pre Challenge	Post Challenge	Pre Challenge	Post Challenge	Pre Challenge	Post Challenge	Pre Challenge	Post Challenge	
C57BL/6 mice	PBS	Pool 1	<10		<10		<10		<10	
		Pool 2	<10	80**	<10	<10**	<10	<10**	<10	<10**
		Pool 3	<10		<10		<10		<10	
	OVX836	Pool 1	<10	320	<10	<10	<10	<10	<10	<10
		Pool 2	<10	320	<10	<10	<10	<10	<10	<10
		Pool 3	<10	320	<10	<10	10	<10	<10	<10
	IIV	Pool 1	<10		20		80		10	
		Pool 2	<10	160**	20	20**	40	40**	10	10**
		Pool 3	<10		10		80		<10	
	OVX836 /IIV	Pool 1	20	160	80	80	40	20	20	10
		Pool 2	<10	160	40	40	40	40	20	20
		Pool 3	20	160	80	80	40	20	20	10
Balb/c mice	PBS	Pool 1	<10		<10		<10		<10	
		Pool 2	<10	160**	<10	<10**	<10	<10**	<10	<10**
		Pool 3	<10		<10		<10		<10	
	OVX836	Pool 1	<10	160	<10	<10	<10	<10	<10	<10
		Pool 2	<10	80	<10	<10	<10	<10	<10	<10
		Pool 3	<10	160	<10	<10	<10	<10	<10	<10
	IIV	Pool 1	<10	160	20	80	160	320	10	20
		Pool 2	<10	160	20	80	160	160	10	10
		Pool 3	<10	80	10	40	80	80	10	10
	OVX836 /IIV	Pool 1	<10	80	40	40	40	40	20	10
		Pool 2	<10	160	40	40	80	80	10	10
		Pool 3	10	160	40	40	160	320	10	20

* Strain used for viral challenge

** Results from one remaining mouse