Article

Historical H1N1 Influenza Virus Imprinting Increases Vaccination Efficacy by Influencing Antibody Longevity and Neutralization Activity in Ferrets

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Abstract: Influenza virus imprinting is now understood to significantly the influence immune responses and clinical outcome of influenza virus infections that occur later in life. Due to the yearly cycling of influenza viruses, humans are imprinted with the circulating virus of their birth year to subsequently build a complex influenza virus immune history but very little is known about how the imprinting strain influences vaccine responses. To investigate the imprinted host immune responses to split-virion vaccination, we imprinted ferrets with a sublethal dose of the historical seasonal H1N1 strain A/USSR/90/1977. After a +60 day recovery period was given to build immune memory, ferrets were immunized and the challenge at Day 123. Samples were collected throughout the time course and immunological assays were performed to investigate recall mechanisms. The preimmune-vaccinated ferrets did not experience significant disease during challenge while naïve-vaccinated ferrets had severe disease. Hemagglutination inhibition assays showed preimmune ferrets had a more robust antibody response post vaccination, increased virus neutralization activity. Virus specific immunoglobulins were of predominantly the IgG isotype suggesting B cell maturity and plasticity at vaccination. These results are important and should be considered for vaccine design.

Keywords: influenza virus; imprinting; haemagglutinin; antibody titre; quadrivalent vaccine; influenza A; H1N1; split-virion; isotype; virus neutralization

1. Introduction

Influenza A virus infection is a recurrent and unsolved public health problem. Two types of Influenza viruses (Orthomyxoviridae), influenza A and influenza B, each with its own subtypes and lineages, currently circulate in humans [1-3]. These viruses cause millions of hospitalizations and thousands of deaths yearly infecting between 5% and 30% of the global population [4-8]. Furthermore, infants, the elderly, pregnant women, and people with pre-existing medical conditions are at higher risk for developing severe disease requiring hospitalization from influenza infection [9].

The well-known yearly cycling of the influenza virus is the result of the reciprocal relationship between the host and virus: the human immune responses influence virus mutation which feeds back to influence immune response [8]. As a result, vaccines need to be reformulated every year, at great

45 expense, to match circulating strains [10]. The changes in the influenza virus occurs through a process
46 known as antigenic drift [11]. Due to antigenic drift, distinct seasonal strains emerge each year with
47 the ability to infect a vulnerable human population [12]. The very first influenza virus exposure in a
48 human during infancy is known to imprint the host immune system leading to virus-specific B cell
49 and T cell memory clones that are long-lived and skewed toward antigenic sites of the imprinting
50 virus [13]. For the humoral arm of the immune system, imprinting leads to antibodies and memory
51 B cells that target first virus epitopes which remain throughout life and may impact future infections
52 [14]. Over time humans build a complex immune history as they are cyclically infected with novel
53 influenza strains and receive seasonal vaccinations [15-17]. It is recognized that the immune
54 responses from the imprinting infection which leads to *preimmunity* significantly influences vaccine
55 and infection outcomes, but the mechanisms that regulate vaccine responses in the
56 imprinted/preimmune host have yet to be elucidated [13,18-20].
57
58 The host's primary infection with an influenza virus initiates a cascade of innate and adaptive
59 immune events that culminates in immunological memory. This first infection in a person's lifetime
60 is referred to as the viral imprinting event [14]. The goal of the immune response is to mobilize
61 adaptive immunity for the production of antibodies capable of virus neutralization. This response is
62 defined by the generation of short-lived plasma cells (antibody producing cells) [21]. Activated B cells
63 mature into plasmablasts and plasma cells, producing antibodies targeted at viral epitopes. The
64 adaptive cellular and humoral responses move through three phases: expansion, contraction, and
65 memory.   The expansion phase is the proliferation of antigen-specific T and B cells producing a large
66 number of reactive cells to control the pathogen. Experimental studies from our group as well as
67 others investigating immune responses in animal models (NHP and ferret) have shown that
68 lymphocytes as well as virus-specific antibodies circulate at high numbers (in the expansion phase)
69 even after pathogen clearance (up to 48 days) [22,23]. Eventually, adaptive immune cells contract
70 until there is a limited number of highly specific cells able to circulate for immune surveillance, which
71 is referred to as the B cell memory pool [24]. Importantly, the memory phase is the time period in
72 which humans are typically re-exposed either through reinfection or vaccination.
73
74 Immune history has an impact on host influenza vaccination responses [14,25,26]. These human
75 studies, based on serology or epidemiological study designs, suggest that understanding how the
76 host interacts with an antigenically divergent pathogen, such as influenza viruses over multiple
77 exposures will be important for identifying susceptible populations and designing the next
78 generation of influenza vaccines. Here we investigated the responses to the split, inactivated virion
79 Sanofi QIV influenza virus vaccine (Sanofi Pasteur, Canada) in preimmune ferrets. We first
80 established preimmunity over 67 days to ensure the ferrets were outside of the expansion phase of
81 the adaptive immune response. Vaccinated ferrets that were previously infected with the historical
82 seasonal influenza virus strain A/USSR/90/1977 had significantly divergent responses in terms of
83 antibody reactions and clinical disease compared to naïve ferrets - vaccinated ferrets. These results
84 have implications on future vaccine design and evaluation.

85 ## 2. Materials and Methods

86 ### Ethics statement
87 All animal work was conducted in strict accordance with the Canadian Council of Animal Care
88 (CCAC) guidelines. The protocol license numbers AUP 5316 and 1031 were assigned by the Animal
89 Care Committee of the University Health Network (UHN). UHN has certification with the Animals
90 for Research Act, including for the Ontario Ministry of Agriculture, Food and Rural Affairs, Permit
91 Numbers: #0132–01 and #0132–05, and follows NIH guidelines (OLAW #A5408-01). The animal use
92 protocol was approved by the UHN Animal Care Committee (ACC). All efforts were made to
93 minimize animal suffering. Infections and sample collections were performed under 5% isoflurane
94 anesthesia.
95

Influenza Virus and animals

The 2009 H1N1 virus strains, A/California/07/2009 (Cal/07), A/USSR/90/1977 (USSR/77), and A/Taiwan/1/1986 (Taiwan/86), were provided by the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA. $TCID_{50}$ and EID_{50} determinations were done as previously described [27]. All virus work was performed in a BSL-2+ facility as previously described [28]. Sanofi FLUZONE® quadrivalent QIV influenza vaccine from the 2015-2016 influenza season was acquired from Sanofi Canada (North York, Canada). The vaccine contained concentrated HA proteins from A/California/07/2009 (H1N1); A/Victoria/210/2009 (H3N2); B/Brisbane/60/2008 (B-Victoria Lineage); B/Florida/04/2006 (B-Yamagata Lineage). Adult female ferrets (aged ~5 months to 1 year) were purchased from Triple F Farms (Gillett, PA, USA). Ferrets were determined to be seronegative by haemagglutination inhibition (HI) assay against currently circulating influenza A and B strains before infection.

Infections and Vaccinations

Ferret were infected intranasally as previously done [29]. Briefly, ferrets were anesthetized and infected with seasonal viruses or pandemic viruses at 10^6 EID_{50}. The volume of inoculum was 1 mL for each ferret (0.5 mL in each nare). For vaccination, ferrets were vaccinated intramuscularly with a whole human dose of FLUZONE® Sanofi QIV vaccine in the upper hind limb. Ferrets were observed post infection or vaccination for adverse effects.

Clinical Monitoring

Weight, temperature, and clinical signs were monitored following infections and vaccinations for 14 days similarly as previously done [30]. Weight and temperature for each day were calculated as a percentage of original values determined on Day 0 and days prior to study initiation. Standard deviation and standard error were calculated for weight and temperature percentages within each group. Clinical signs (body temperature, body weight, level of activity, nasal discharge, and sneezing) were observed daily for 14 days pi, post-vaccination, or pc. Ferrets were examined at the same time each day for consistency. Nasal discharge, sneezing, and inactivity was observed and recorded but not reported here.

Viral Titers, HAI Titers, Microneutralization titers (MN), and IgG/IgM relative isotype levels.

Viral titers were calculated from collected nasal wash samples pc. Nasal washes were subjected to a TCID50 assay followed by HA assay against virus strain of interest to determine viral titers. Viral load was calculated using the Reid and Meunch method [31]. HAI titers were determined in serum collected throughout the entire study by HAI assay using USSR/77, Cal/09, or Taiwan/86 live viruses or using the WHO circulating influenza virus detection kit (2016-2017). The WHO kit assays for H1N1 2009, circulating H3N2, circulating B-Yamagata, and circulating B-Victoria strains. Microneutralization titers (MN) results were evaluated by enzyme-linked immunosorbent assay (ELISA). Neutralizing antibody titers were determined by the highest dilution of RDE-treated anti-sera that disrupted infection (100 $TCID_{50}$) on MDCK cells at the reading lower than 50% signal reading measured from virus+cell and cell only controls. The determination of IgG and IgM specific antibodies in collected serum was based on enzyme-linked immunosorbent assay (ELISA) technique as previously described [32] . Briefly, ELISA plates were directly coated with A/California/07/2009 overnight at room temperature. Plates were washed with PBS containing 0.05% Tween 20 (T-PBS) and blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C. Antigen-coated plates were incubated with 1:1,000-diluted serum samples overnight at 4°C. After washing with T-PBS, plates were incubated with goat anti-ferret immunoglobulin (IgM and IgG) horseradish peroxidase (HRP) conjugates (Rockland Immunochemicals) in a 1:10,000 dilution for 2 h at 37°C. The reaction was developed by *o*-phenylenediamine for 30 min, and the optical density was read at 492 nm.

Histopathology

148   Lungs were collected at necropsy, perfused with formalin and paraffin embedded. Following
149   sectioning the tissues were mounted and H&E stained. High resolution scans were performed using
150   an Aperio ScanScope XT (Leica Biosystems) at 40x magnification. Images were captured using the
151   HALO program from UHN AOMF (Advanced Optical Microscopy Facility) at 5x, 10x, or 20x
152   magnification of the scan.
153
154   **Statistical Analysis**
155   ANOVAS or Student's t-test was used to determine p-values when comparing groups.   The
156   inactivity index of each viral infection was calculated using the scores observed daily. For both the
157   viral and antibody titers, standard deviation was calculated within each experimental group.
158   Student's t-test or ANOVAS was used to determine the p-value among groups.
159

160   **3. Results**

161   *3.1. Study Design*

162   With the exception of neonates and young infants, the majority of the human population has been
163   previously exposed to influenza viruses. By living through the winter influenza season or any time
164   year-round in the tropics, people will be exposed to influenza viruses either from community
165   transmission or vaccination; therefore, understanding vaccine responses in the preimmune host will
166   give a more accurate insight into the responses to vaccination for the majority of the population. The
167   early stages of influenza infection are defined by the antiviral response and innate immune cell
168   activity [33]. Subsequently, adaptive cellular and humoral responses move through phases of
169   expansion, contraction, and memory, to establish host long-term protection (**Figure 1a**) [24].   To
170   understand how the preimmune host responds to vaccination, we designed a study using the ferret
171   model to build an influenza preimmune background for subsequent vaccination and challenge. The
172   design and experimental timeline of the study are shown in **Figure 1a**. The key to our study design
173   was the elongated recovery period. Previous studies investigating the immune re-encounter with
174   influenza viral antigens or live virus infection are frequently designed with short recoveries of 14 to
175   28 days separating primary and secondary challenges [34-36]. These time frames do not properly
176   account for the actual immunological recovery. Our rationale for this study was to allow the ferrets
177   to recover over several months. Several studies have shown that influenza virus-specific adaptive
178   immune cells exist in high numbers in circulation even after 21 days post infection [22,23].   Our
179   design includes a long recovery period so that re-exposure (infection or vaccination) occurs after the
180   contraction phase to more accurately reflect the seasonal exposures of influenza viruses in humans.
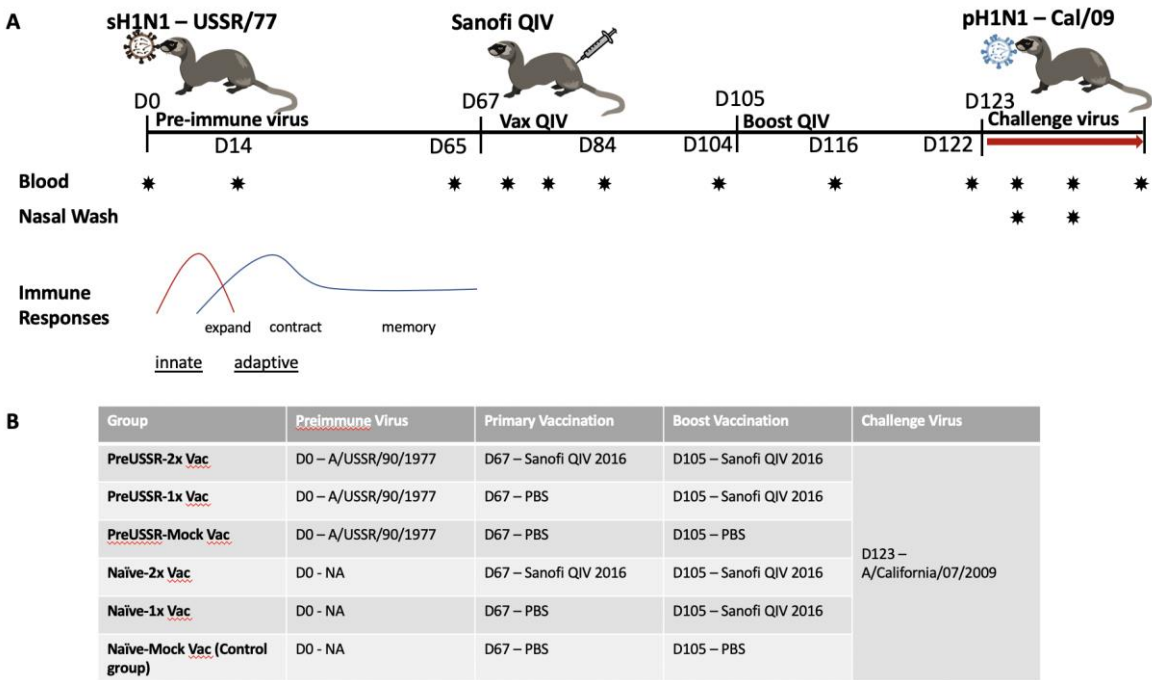
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182

183

Figure 1. Study timeline and experimental group design for the investigation of the influence of influenza immune history on vaccines responses to Sanofi FLUZONE® QIV.

184   To establish preimmunity, adult ferrets were infected (imprinted) with a sublethal dose of the
185   historical seasonal H1N1 A/USSR/90/1977 (USSR/77) Day 0 of the study. After imprinting and
186   development of antibodies specific for the imprinting virus, we consider the host to be *preimmune* to
187   the influenza virus. USSR/77 was chosen as the imprinting virus since it was a previously circulating
188   human influenza virus that would have significantly impacted the immune history of many people
189   alive today [37]. Furthermore, USSR/77 is a virus that re-emerged in the 1970s and is antigenically
190   divergent compared to our proposed contemporary challenge virus [13]. Ferrets recovered over 67
191   days to decrease non-specific immune responses. The ferrets were subsequently vaccinated at Day
192   67 post-imprinting infection (pi) and boosted on Day 105 pi with the Sanofi QIV split virion vaccine
193   (FLUZONE® Sanofi-Pasteur, PA, USA). To control the experiment, 6 groups (6 ferrets per group)
194   were designed: **1.)** preimmune USSR/77 – QIV vaccinated and boosted (preUSSR-Vac2x); **2.)**
195   preimmune USSR/77 – QIV vaccinated with no boost (preUSSR-Vac1x); **3.)** preimmune USSR/77 –
196   mock vaccinated (preUSSR-Mock Vac); **4.)** naïve – QIV vaccinated and boosted (Naïve – Vac2x); **5.)**
197   naïve – QIV vaccinated with no boost (Naïve – Vac1x); and **6.)** naïve - mock vaccinated (Naïve – Mock
198   Vac) (**Figure 1b**). To investigate protection from vaccination or preimmune infection, ferrets were
199   infected with a currently circulating 2009 H1N1 pandemic virus on D123 pi. This virus,
200   A/California/07/2009 (Cal/2009), is one of the components of the Sanofi® QIV vaccine used in our
201   study. Furthermore, this strain is a representative from the 2009 H1N1 influenza pandemic which
202   marked the emergence of an antigenically novel H1N1 "lineage" [38]. Since the vaccine contained
203   Cal/2009 antigen, vaccinated animals should be protected against challenge with Cal/2009.
204   Considering the USSR/77 infection and Cal/09 reinfection, this combination represents a
205   monosubtypic heterologous reinfection. Importantly, these viruses do not produce cross-reacting
206   HAI antibodies when infected into the naïve ferret model as we have previously shown [37]. The
207   protein alignment in the highly variable region of HA (135-295aa), which belongs to the HA-RBD
208   area, has only 59% homology between Cal/09 and USSR/77 [37] and this combination of preimmune
209   exposure and challenge virus infection represents conditions plausible for the immune history of
210   people alive today. Blood (serum), lungs, and nasal wash were collected at specified time points to
211   examine the immune response. Specifically, blood samples were collected at Days 0, 14, 65, 68, 72, 79,

212   104, 116, 122 pi and pc at Days 2, 7, and 14. These time points were chosen as they represented
213   significant days surrounding the infection and vaccination events, as we have previously shown
214   [28,32].

215   *3.2. Milder clinical disease was observed in the preimmune-vaccinated ferrets at challenge*

216   Three groups of ferrets were infected with 1×10^6 EID$_{50}$ of the historical H1N1 strain USSR/77 at the
217   start of the study and the temperature and weights were monitored in the ferrets over 14 days post
218   imprinting (pi) (**Figure S1**). The remaining ferrets in other groups were left in control rooms to be age
219   matched for subsequent time points in the study. Analysis of the temperature and weight change of
220   each group indicated minimal variation between groups following imprinting infection. All groups
221   lost a moderate amount of weight following infection. At nadir, all groups had lost between 5 and
222   10% of their original weight similar to our previous findings with this virus [37]. All groups had a
223   temperature increase peaking Day 2 pi. On Day 67 and Day 105, the ferrets were vaccinated and
224   boosted, respectively, as specified by their group (**Figure S2**). Minimal weight loss or temperature
225   change was observed over a 14-day monitoring period post vaccination and boost similar to monitor
226   for reactogenicity.

227   To determine how well the vaccine was able to protect against disease at challenge, on Day 123 the
228   ferrets were challenged with an infectious dose of 10^6 EID$_{50}$ of A/Cal intranasally. The ferrets were
229   monitored for signs of clinical disease including weight loss, fever, and lethargy post challenge (pc).
230   The preUSSR-Vac2x group lost minimal weight. Maximum weight loss was observed on Day 2 pc
231   where weight dropped to 97% of original weight (**Figure 2a**). Furthermore, the preUSSR-Vac2x did
232   not have a temperature increase pc, but instead experienced a slight drop in body temperature
233   (**Figure 2b**). Mild disease was also experienced by the preUSSR-Vac1x group although the time
234   period at which this group had weight loss was longer than that seen in the preUSSR-Vac2x group
235   and maximum weight loss was noted at 96% of original weight. No temperature increase was
236   observed in the preUSSR-Vac1x group. Conversely, the naïve-Vac2x and naïve-Vac-1x groups both
237   experienced significant temperature increase to 105% and 103% of original temperature at Day 2 pc,
238   respectively ($p<0.05$ by t-test compared to preUSSR-Vac2x). These groups, as well as the control
239   group (naïve-MockVac), had significant weight loss compared to the preUSSR-Vac2x group.
240   Interestingly, the naïve-Vac2x group had the largest amount of weight loss pc where maximum
241   weight loss was seen on Day 9 pc at 82% of original weight. Together, these results suggest that
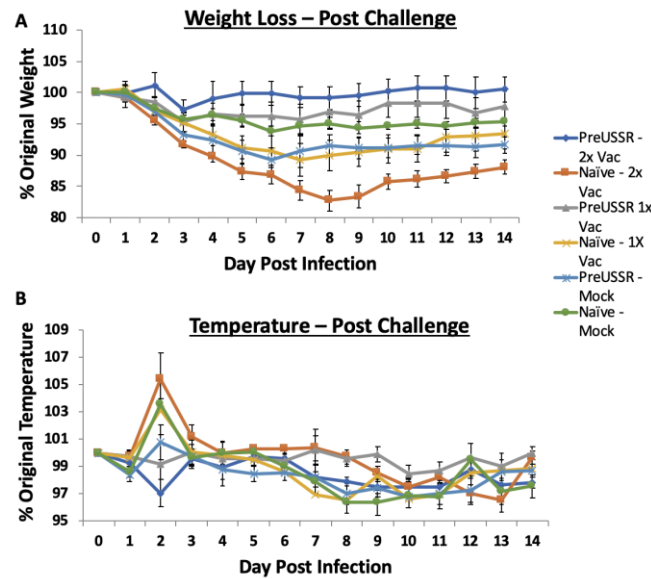242   preimmune ferrets had a greater vaccination efficacy as shown by a milder clinical disease.

243

Figure 2. Prior exposure to a heterologous H1N1 influenza virus increases influenza vaccine protection.
Ferrets were infected with USSR/77 (10^6 EID_{50}) to establish an influenza-specific immune background or were left naïve. After 67 and 105 days, select groups of ferrets were vaccinated with the Sanofi FLUZONE® QIV vaccine and challenged at Day 123 with the Cal/07 H1N1 pandemic 2009 virus. (**a**) Weight loss and (**b**) temperature changes were monitored for 14 days pc. * indicates a p-value of less than 0.05

244 *3.3. Decreased respiratory infection in preimmune-vaccinated ferrets*

245 Hallmarks of influenza virus induced illness include respiratory virus shedding and lung pathology.
246 To determine the viral susceptibility and respiratory disease, we collected nasal washes (NW) and
247 lungs from ferrets pc with the Cal/09 H1N1 pandemic virus. NW were collected Days 2 and 7 pc.
248 Virus was only detected in NW collected on Day 2 pc and is shown in **Figure 3**. Titers in the pre-
249 USSR-vaccinated groups were markedly or significantly lower than the naïve-vaccinated or naïve-
250 unvaccinated control groups. Both preUSSR-vaccinated groups had viral titers averaging at 2.5 $\log_{10}$
251 $TCID_{50}$. Furthermore, the naïve-Vac2x group had the highest titer of viral shedding (**Figure 3**).
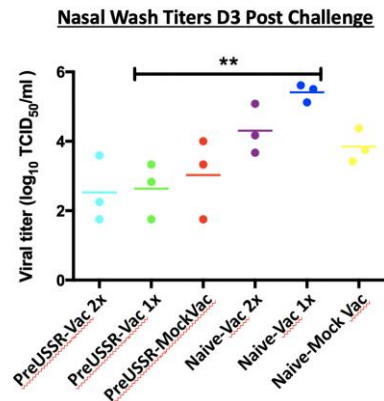

Figure 3. Viral shedding was lower in previously infected and vaccinated hosts.
Nasal washes were collected from all groups post Cal/09 challenge on Day 2 pc. Live viral load was calculated by $TCID_{50}$ titration assay using MDCK cells. Student's t-tests were performed among groups to determine statistical significance. ** indicates a p-value of less than 0.001

252 Histopathological analysis of the lungs collected on Day 14 pc with Cal/09 suggested distinct
253 respiratory responses dependent on influenza immune background. Lungs were collected at
254 necropsy and prepared for histopathological analysis (H&E staining). Stained and mounted lung
255 sections were viewed at High (20x) and Low (5x) Magnification (**Figure 4**). The Naïve-MockVac
256 group showed typical Day 14 pathological features of Cal/09 infection including mononuclear cell
257 infiltration, bronchiolar wall thickening, and hemorrhage (left panels)[31]. The preUSSR-MockVac
258 lungs had evidence of necrotising alveolitis (middle left panels).   The preUSSR-Vac2x group ferret
259 lungs had evidence of either mild pathological changes with minimal leukocyte infiltration,
260 peribronchiolar thickening, and alveolar wall thickening (mild) or significant pathological features
261 of interstitial pneumonia with alveolar wall thickening, edema, and fibrosis (affected) (right panels).
262 Taken together, the analysis of viral load shedding and histopathology suggested that the outcomes
263 in the respiratory tract were dependent on the immune background of combinations of vaccination
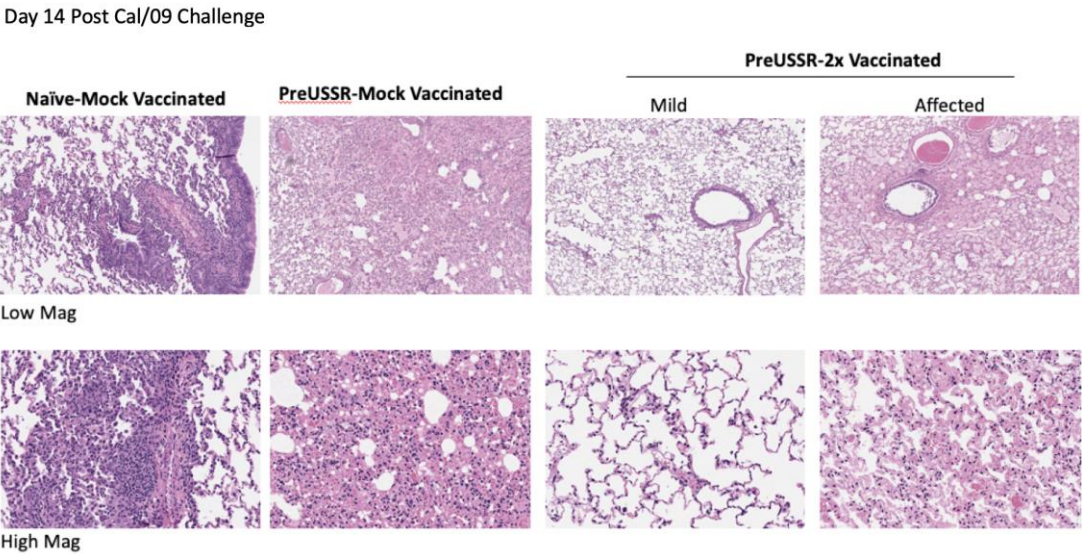264 and pre-infection.

Figure 4. Histopathological analysis shows differential lung pathology dependent on pathogen and vaccination history. Harvested lungs from all ferrets were processed for histopathological assessment. Tissue morphology was assessed by hematoxylin & eosin staining. Data was collected from at least three ferrets per group and results are a representative of the inoculations/infections. High resolution scans were performed using an Aperio ScanScope XT (Leica Biosystems) at 40x magnification. Images were captured using the HALO program from UHN AOMF (Advanced Optical Microscopy Facility) at 5x (low) or 20x (high) magnification of the scan.

265

266 *3.4. Humoral immunity is differentially regulated in the preimmune host*

267 Serum antibody titers reactive toward the HA protein are often indicative of how protected an
268 individual will be toward a specific influenza virus strain. We next investigated the HA specific
269 antibody responses in each experimental group over the experimental time course to determine if
270 influenza background influences the antibodies elicited following vaccination. Sera was collected
271 from the experimental groups along the experimental timeline on Days 0, 14, 65, 84, 104, 122, 125,
272 130, 137. Standard HAI assays were performed to quantify antibody specificity and regulation against
273 influenza HA antigens (**Figure 5** and **6**).   USSR/77 virus was used to quantify antibodies reactive to
274 this antigen, whereas the 2016 WHO kit was used to quantify antibodies toward the vaccine antigens:
275 2009 H1, H3, B-Yamagata, and B-Victoria antigens.

276 Heat maps of the calculated HAI units were generated to visually summarize the HAI data against
277 seasonal USSR H1, pandemic 2009 H1, H3, B-Yamagata, and B-Victoria HA antigens over the 137-
278 day time course (**Figure 5**).   HAI units for each sample time point is also depicted to give a more
279 detailed view of the changes in elicited antibodies over time (**Figure S3**). The mean HAI units per

280 group per time point was calculated over the entire study. The H1-USSR heat map (top left graph)
281 shows strong HA antibody generation by Day 14 following infection for all infected groups. All
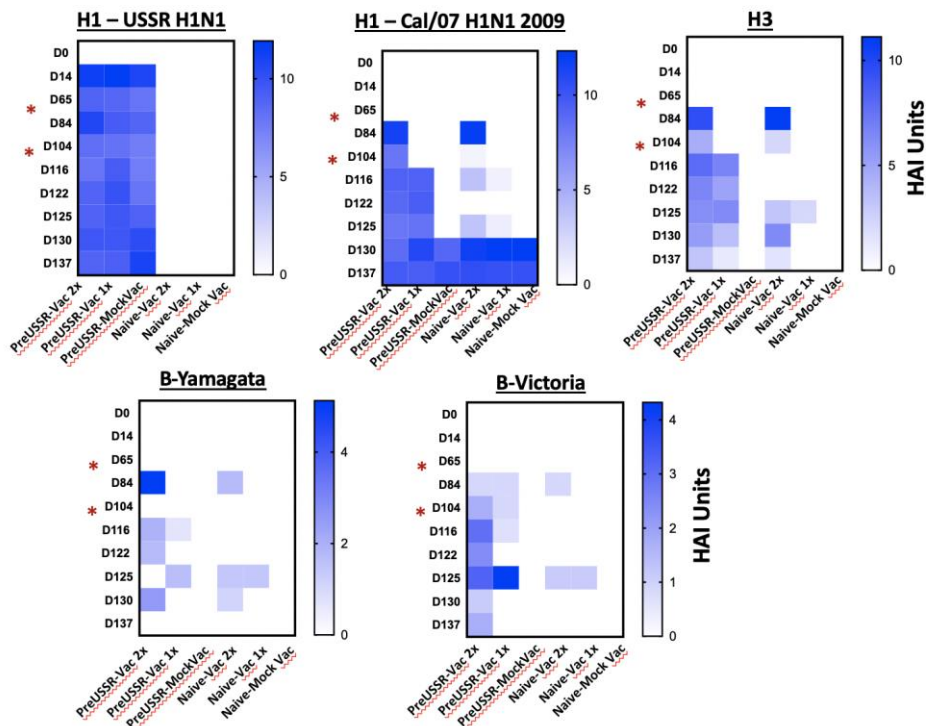282 ferrets infected with USSR/77 (H1-USSR) developed HA binding antibodies to the H1-USSR antigen

Figure 5. Increased vaccine antibody titer in previously infected ferrets.
The mean HAI U for each experimental group was calculated and the results were plotted in heat maps to visually represent changes in antibody titer and specificity over time. HAI assays were performed using specific whole virus (USSR/77) or BPL inactivated vaccine antigens and turkey red blood cells. Previously infected ferrets produced antibody responses of greater titer and longer lived in circulation compared to naïve-vaccinated ferrets. Red arrow indicates vaccination days.

283 between 10 and 12 HAI Units as assessed on Day 14 pi.   Titers in these previously infected animals
284 remained between 7 and 12 HAI Units for the entire study. The uninfected ferrets remained negative
285 throughout the study. At vaccination and boost (Day 67 and Day 105, respectively), the USSR infected
286 ferrets had strong HA antibody responses shown by high HAI titers to the H1 2009 HA protein (top
287 middle graph). These H1 2009 antibody titers generated following vaccination in the preUSSR group
288 remained high throughout the time course. Following first vaccination, both previously infected and
289 naïve ferrets developed high antibody tiers to the H1 2009 HA (H1-2009), between 10 and 14 HAI
290 Units, shortly after vaccination (Day 14).   When titers were quantified in sera collected at a longer
291 time point (30 days) following vaccination, the preimmune ferrets had sustained antibody values
292 between 6 and 10 HAI Units whereas 4/5 of the naïve-vaccinated ferrets had undetectable levels of
293 H1-2009 reactive antibodies.   Fourteen days following boost, the preUSSR-Vac2x ferrets had H1-
294 2009 titers between 8 and 10 compared to lower values seen in the naïve-Vac2x ferrets (between 3
295 and 4 HAI Units).   Furthermore, H1-2009 antibody titers assessed prior to vaccination showed no
296 detectable levels and between 8 and 11 for the naïve-Vac2x and preUSSR-Vac2x ferrets, respectively,
297 suggesting no cross-reactivity was detected by HAI for antigens Cal/09 and USSR/77. The naïve-
298 Vac1x ferrets also had strong or moderate responses to the vaccine directly following vaccination but
299 these titers quickly waned and were not maintained over time. Interestingly, these trends were
300 consistent for the other HA antigens H3, B-Yamagata, and B-Victoria, where the preUSSR ferrets had
301 a stronger antibody response following vaccination and boost despite the antigenic divergence of the
302 vaccine antigens with the original imprinting virus.   Taken together, these results suggest that a
303 previous infection to the influenza viruses primes the naïve immune system to have greater antibody
304 responses following vaccination with the Sanofi QIV® vaccine administered by intramuscular
305 injection.

306   To visualize numerical changes in antibody titers across the experiment for the H1 2009 antigen, HAI
307   values were determined for each ferret and the group averages were calculated per time point and
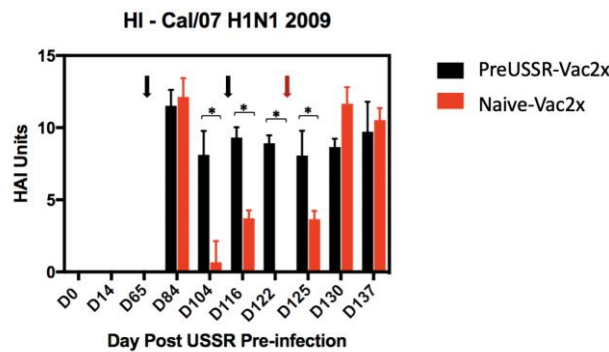308   plotted in a histogram focused on only the preUSSR-Vac2x and the naïve-Vac2x groups (**Figure 6**).

Figure 6. Focused titer analysis of the H1-2009 specific antibody titers shows specific antibody regulation dependent on previous influenza virus exposure.

HAI values of only the preUSSR-Vac2x group was compared numerically by histogram to the naïve-Vac2x over the entire time course against the 2009 H1N1 pandemic Cal/09 virus to focus the analysis on the differences caused by immune background/pre-immunity. Black arrows denote time of vaccination. Red arrows denote challenge day. Day 0 is the time of infection with the seasonal H1N1 USSR/77 virus. Values are graphed in HAI Units.

309   Serum collected on Day 84 following first vaccination showed similar HAI levels between the two
310   groups (>11 HAI Units).   Interestingly, the subsequent blood collection prior to boost indicated a
311   significant drop in 2009 H1 antibody titer in the naïve-Vac2x groups to <2 HAI Units whereas the
312   PreUSSR-Vac2x group had a minimal drop in HAI to 8 HAI Units. Statistical differences in HAI titers
313   for the 2009 H1 antigen between the preUSSR-Vac2x group and the naïve-Vac2x group were seen
314   throughout the time course until Day 7 pc.

315   *3.5. Mature B cells play a role in the vaccine responses of the imprinted host*

316   Above, we found that the ferrets previously exposed to an antigenically divergent influenza virus
317   had stronger antibody responses to Sanofi QIV® vaccination. From these findings, we next
318   investigated the dynamics of the antibody isotype produced during the sequential infections and
319   vaccinations of our study. To do this, we performed virus specific IgG and IgM ELISAs to quantify
320   the isotype responses specific for the Cal/09 virus. Serum collected from all time points were
321   evaluated but only the time points prior to challenge and following challenge (D2, D7, and D14) are
322   shown (**Figure 7**). No statistical differences were found in the IgM levels among each group
323   throughout the time course with the exception of the naïve-MockVac group did not have any virus
324   specific IgM antibodies until after challenge (left graph) as expected. All IgM titers remained
325   oscillating around 0.5 OD 492 nm. Quantification of virus-specific IgG production found statistical
326   differences in IgG levels among the groups Day 2 and Day 7 pc (right graph). Specifically, the
327   preUSSR-Vac2x group had statistically higher IgG levels (OD =~1) than all other groups, which had
328   low values close to 0. Naïve-MockVac group were negative for IgG antibody production until after
329   challenge as expected. Interestingly, the preUSSR-MockVac group had significant increases in IgG
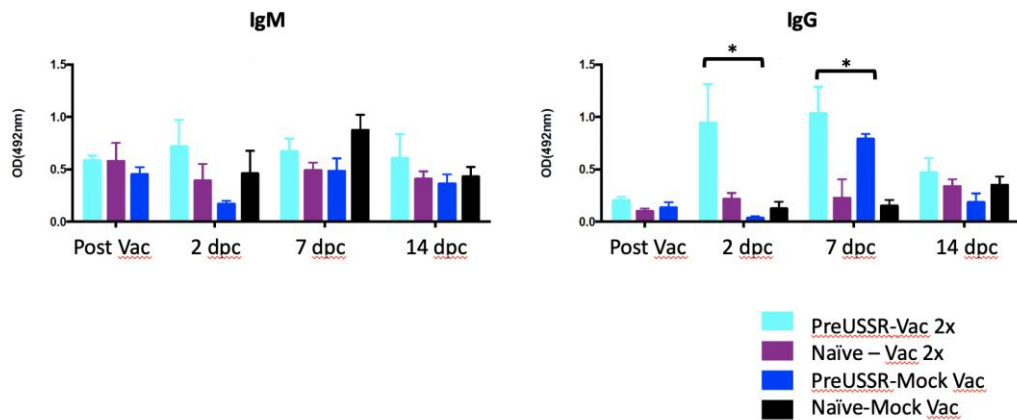330   on Day 7 following infection.

331

Figure 7. Virus specific IgG isotype antibodies were predominant in the preUSSR-Vac2x group.
Isotype ELISAs were performed using the serum collected from each group throughout the time course to determine the proportion of virus-specific IgG or IgM antibodies produced. Only time points post-vaccination and challenge are shown. Student's t-test was conducted to compare with results on Day 0, *p<0.05.

332   Since the dominant antibody response was of the IgG isotype that is indicative of a more mature B
333   cell response, we were interested in determining the temporal generation of vaccine antigen-specific
334   antibodies at early time points following vaccination. We collected serum in the early time points
335   following vaccination (Days 0, 3, 7, and 14) from naïve ferrets, ferrets previously infected with
336   USSR/77, and naïve-mock vaccinated ferrets as control (**Figure 8a**). HAI assays were performed with
337   the serum against the 2009 H1 antigen and showed that all groups were negative for 2009 H1 HAIs
338   at Days 0 and 3 suggesting that cross-reactive HAI antibodies to the 2009 H1 HA head were not
339   present at these times. By Day 7 the preUSSR-Vac group had a positive HAI response to 2009 H1,
340   reporting at ~5 HAI Units (light blue bars), whereas both the vaccinated alone and mock vaccinated
341   group did not have a positive HAI.   Furthermore, by Day 14 the preUSSR-Vac group had a strong
342   HAI response at 9 HAI Units compared to the naïve-vaccinated ferrets which had a mean of 3 HAI
343   Units for the group (light purple bars). These results suggest that the ferrets with a previous but
344   antigenically divergent H1N1 infection generated a faster and stronger response to the new 2009 H1
345   antigen.

346   To investigate the possible mechanism leading to greater protection in the preimmune-vaccinated
347   ferrets, we next investigated the function of the antibodies produced over the time course by
348   performing microneutralization (MN) assays (**Figure 8b**). Using serum collected pre-challenge, MN
349   were performed as previously described against the Cal/09 virus [28]. Pre-challenge serum (Day 123)
350   was used for the MN assays as an indicator or predictor of clinical disease observed during challenge.
351   The preUSSR-Vac2x group had the highest titer of MN antibodies at ~10, which were statistically
352   greater than any other experimental group. Furthermore, both the preUSSR-MockVac and the naïve-
353   Vac2x had lower MN titers compared to the preUSSR-Vac2 group but similar MN titers to each other.
354   As expected, there were not any MN titers detected for the naïve-mock group. Together, these data
355   show that the preUSSR-Vac2x group had the highest titer of functional antibodies capable of
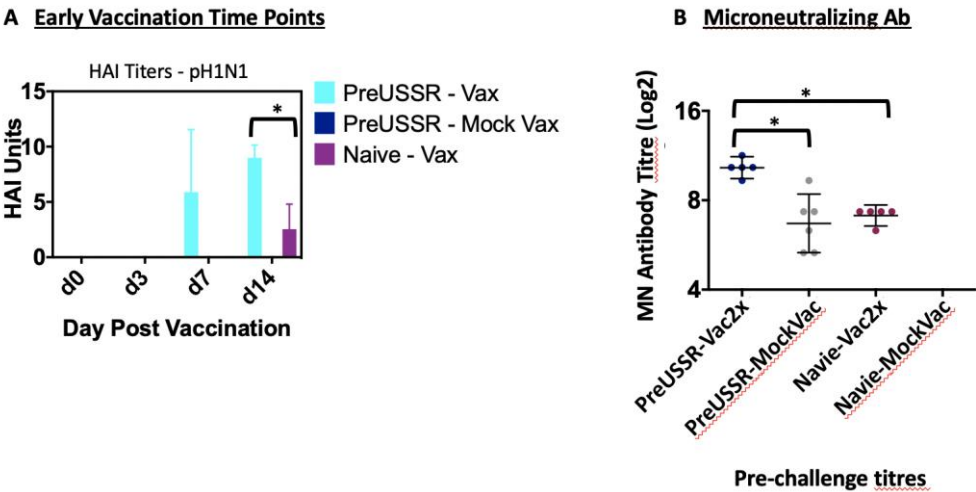356   inhibiting viral infection.

357

Figure 8. PreUSSR-Vac2x ferrets had specific antibody function and dynamics following vaccination. Serum was collected at early time points post vaccination (Days 0, 3, 7, and 14 post vaccination). (a) HAI assays were performed as previously described on serum against the H1 antigen to determine the early dynamics of the antibody response after vaccination. (b) Microneutralization assays were performed by standard protocol on MDCK cells using serum collected prechallenge. Student's t-test was conducted to compare with results on Day 0, *$p<0.05$.

358   Previously, sequential infections with divergent influenza viruses leads to the elicitation of the
359   antibodies that are more broadly reactive toward a larger spectrum of antigenically distinct influenza
360   strains [15,39,40]. Although the induction of broadly neutralizing antibody generation was shown to
361   be through an HA stem mechanism, we were interested in knowing whether the sequential exposure
362   to these viruses had an effect on the specificity and cross-reactivity of the antibodies produced.
363   Therefore, we performed additional HAIs using the antigenically distinct influenza strain
364   A/Taiwan/1/1986 (Taiwan/86) with our serum samples. Taiwan/86 is antigenically removed from
365   both USSR/77 and Cal/09 and has 90% homology to USSR/77 and 62% to Cal/09 as analyzed by the
366   HA receptor binding domain area (a.a. 135-295)[37]. Previously we showed that direct infection with
367   Taiwan/86 does not elicit cross-reactive antibodies toward either USSR/77 or Cal/09 viruses [37]. In
368   this study we also showed that the reverse was true as well, infection with USSR/77 or Cal/09 did not
369   elicit cross-reactive antibodies toward Taiwan/86 as determined by HAI. These results suggest that
370   any cross-reactivity observed in the sequential infection study would be elicited from repeated
371   influenza antigen exposure. An HAI assay was performed using serum collected at Day 14 pc (Day
372   137 of the entire study) since this sera would be representative of the largest number of sequential
373   exposures: PreUSSR->QIV->QIVBoost->Challenge. The assay was performed against the Taiwan/86
374   virus and the results showed distinct trends in HAI cross-reactivity related to the sequence and
375   multiplicity of exposures. Specifically, the preUSSR-MockVac ferrets had the highest titer of cross-
376   reactive antibodies against Taiwan/86 at 9 HAI Units (**Figure 9**). Both vaccinated groups had
377   successively lower titers showing an inverse relationship to the number of repeated exposures.
378   Specifically, the preUSSR-Vac1x and preUSSR-Vac2x had average group titers of 7 and 6 HAI Units,
379   respectively. This data suggested that a low number of sequential exposures could elicit a high titer
380   of cross-reacting antibodies to a highly divergent strain but the more influenza exposures that occur
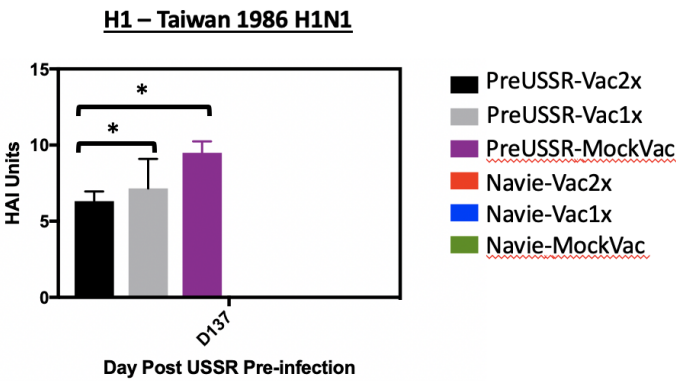381   afterward will refine the response.

382

383

Figure 9. Decrease in cross-reactive antibody titer over sequential influenza virus exposures of antigenically divergent strains.

Serum was collected at end day (Day 137) from all ferrets. Serum was then used for HAI assays against an antigenically divergent influenza virus strain (A/Taiwan/1/1986, Taiwan/86) as a read-out for the development of cross-reactive antibodies. Standard HAI assays were conducted with turkey RBCs against Taiwan/86. Student's t-test was conducted to compare results with the PreUSSR-Mock Vac group, *$p<0.05$.

384

385   **4. Discussion**

386   Every year the influenza vaccine has variable effectiveness against the seasonal circulating
387   viruses allowing continual circulation in humans. Due to continual circulation and influenza virus
388   exposure, humans have a complicated influenza background. The split virion vaccine is the vaccine
389   most often used for seasonal influenza vaccination worldwide, but there has been limited analytical
390   and experimental investigation in animal models imprinted with historical or contemporary human
391   influenza viruses. To investigate the imprinted host-responses to the Sanofi QIV® vaccine, we
392   developed a ferret model with the USSR/77 influenza virus background for vaccine investigation
393   during the immune memory phase of the imprinting infection. We found that the imprinted ferrets
394   had increased antibody responses to vaccination that led to increased protection from a divergent
395   challenge virus. Interestingly, although the naïve ferrets were capable of eliciting an antibody
396   response to the vaccine, they developed significant disease with higher viral titers. Together, our
397   results suggest that much could be learned from the immune responses to vaccination in the
398   preimmune host and that the mechanisms identified could be leveraged for improving vaccines.
399
400   Here our results suggest that previously infected ferrets are better immunologically equipped to
401   respond to the split virion influenza vaccine. We showed that the ferrets preimmune to the USSR/77
402   virus were able to mount a greater and longer sustained antibody response as determined by HAI
403   assay toward all vaccine antigens. Furthermore, the antibodies elicited had greater neutralization
404   activity suggesting that the mechanism of milder disease at challenge was the ability to develop more
405   functional and viral infection inhibiting antibodies. This is an important result, especially since the
406   naïve-vaccinated ferrets developed a more severe disease compared to other groups. Furthermore,
407   these results suggest that vaccination on a naïve background may lead to the development of
408   antibodies that are not as efficient for inhibiting viral infection. Infants are the most immunologically
409   naïve population in respect to previous influenza virus exposures. Infants also have been shown
410   countless numbers of times to be at a greater risk for developing severe complications requiring
411   hospitalization following influenza virus infection [9]. The higher attack rates in this age group is

thought to be partially due to the decreased protection offered from vaccination and it is speculated that the influenza naïve infant immune system is the cause of poor vaccine responses [41]. Our data indicating decreased protection following vaccination in naïve ferrets reflects the ineffectiveness of influenza vaccination in naïve infants suggesting the host may need to be primed prior to vaccination. Since the split virion vaccine was not able to elicit a high level of neutralizing antibodies in naïve ferrets as what was seen in the preimmune ferrets, this suggests that the digested viral antigens of the vaccine is not the most appropriate for inducing functional antibodies. Understanding how a prior infection primes the immune system for more effective vaccinations may offer new strategies for priming the immune system in the naïve hosts. More work is needed to elucidate the molecular mechanisms of antigen elicitation of neutralization antibodies for the development of more effective vaccine platforms.

Our findings also showed that there was an increased disease associated with vaccination on a naïve background. Taken together with our conclusions from the previously exposed and vaccinated group, this data suggested that unprimed individuals may be improperly imprinted during vaccination. Improper viral imprinting by vaccination has also been observed for the respiratory syncytial virus vaccine but at a greater clinical cost [42,43]. In the 1960s, a formalin-inactivated RSV vaccine was developed and administered to young children. Unfortunately, the formalin inactivation step inhibited subsequent cytoplasmic processing of the viral antigens upon vaccination. The consequence of improper vaccine imprinting was that the vaccinated naïve children developed an ineffective immune response and subsequent infection with RSV led to enhanced disease. Specifically, enhanced disease was dominated by a Th2 response and the vaccine-elicited antibodies were inefficient at neutralizing the virus [42]. Our observation that the naïve-vaccinated ferrets had decreased production of virus neutralizing antibodies suggested that antibodies elicited in the naïve hosts following vaccination may be less functional that those elicited in the preimmune-vaccinated host. Further investigation is needed to determine how to properly imprint the host during vaccination to elicit antibodies that are optimal at inhibiting viral infection.

During influenza virus infection, a large and diverse pool of antibodies are initially produced and circulated [18]. The majority of these are directed toward the HA molecule [44,45]. The initial humoral response to infection involves the generation of short-lived plasma cells (low-affinity antibody producing cells) that reside in secondary lymphoid organs [21]. These cells expand rapidly, then decline prior to memory establishment. In the germinal centre, B cell specificity is refined for improved responses toward the pathogen. Refinement occurs through a process called affinity maturation. Affinity maturation includes somatic hypermutation (SHM), class-switch recombination (CSR), and clonal selection [46]. Our results from the antibody isotype ELISAs and early time point HAIs showed that preimmune ferrets had a quicker development of vaccine antigen specific antibodies following vaccination and a predominant phenotype of the IgG isotype. The dominance of the IgG isotype suggested the presence of a pre-existing B cell with either existing vaccine antigen specificity or the presence of a pre-existing B cell with the flexibility to modify antigen specificity. Further investigation of the affinity maturation process during sequential exposure of antigenically diverse antigens should be approached to understand how much of a role affinity maturation plays in continual influenza virus infection and vaccination. On the other hand, outside of B and T cell specific responses, there are other immune mechanisms that may be responsible for the faster generation of antibodies following vaccination in the preimmune host as we observed. Specifically, trained immunity is the adaptation of innate immune components after pathogen stimulation. Trained immunity mainly involves the modification of cells such as NK cells, monocytes, and macrophages to produce a "memory" response which can be mobilized over a longer period of time for a second encounter with a specific pathogen [47]. It is possible that either trained immunity or memory B cell dependent responses influenced the specificity of the elicited antibodies after vaccination in the preimmune hosts; however, considering our long rest period between infection and vaccination, trained immunity is less likely. Further investigation of mechanisms of B cell

464 memory and refinement compared to trained immunity will be important to determine the immune
465 mechanisms. Understanding the roles of both mechanisms of memory B cells and trained immunity
466 in the preimmune host's responses to vaccination may be critical for developing the next generation
467 of influenza vaccines.
468
469 Due to the nature of the drifting and shifting influenza viruses, there has been much work
470 investigating immune responses in infection-reinfection animal models. Since the emergence of the
471 pandemic 2009 influenza virus, there have been several experimental and human investigations on
472 monosubtypic heterologous infection-reinfection due to the occurrence of this infection sequence in
473 the human population. In summary, the general finding from these studies was that primary infection
474 with seasonal H1N1 from the 1977 H1N1 lineage followed by an infection with pandemic 2009 virus
475 leads to partial protection with reduction in disease clinical severity in infected individuals [48-53].
476 We previously reported that primary infection induced pre-existing non-HA antibodies during 2009
477 H1N1 virus challenge due to sequence homology with the less immunogenic internal viral proteins.
478 Other reports showed this sequential infection leads to the generation of both HA head and stalk
479 directed antibodies and that stalk antibodies may be responsible for a more broadly protective
480 antibody response [15,39,40,54]. Together, these reports suggest that antibodies generated toward
481 internal proteins and/or the HA stem may also play a role in the clinical outcome we observed.
482 Although we can glean information on the immune responses in the pre-exposed hosts from the
483 experimental infection-reinfection studies, it is important to note that our study involved sequential
484 viral antigen exposure both in the forms of live viral infection and inactivated viral antigens in the
485 split virion vaccine. Our study specifically showed a broader antibody responses in the preimmune
486 ferrets directed toward the HA head as shown in our HA assays which was not recognized in these
487 other studies. From our data we are the first to show evidence for a HA head directed protection
488 which may be due to the digested viral antigens of the vaccine. Future investigation of the antibodies
489 elicited toward the HA stem and viral internal proteins using the long recovery model should also
490 be done similarly to the live viral sequential infection studies [39,40] to gain a more complete picture
491 of the humoral immune responses in the preimmune host during vaccination.
492
493 Our study specifically investigated the vaccine responses in hosts that had a previous seasonal
494 H1N1 1977 lineage virus infection and then a 2009 pandemic H1N1 virus challenge following
495 vaccination. Since these viruses are of the same subtype but different lineage, it is unknown how
496 vaccines responses may be regulated on a different influenza virus background. Each influenza
497 season is dominated by a specific circulating subtype or lineage, suggesting that the imprinting
498 exposure event of a person may be equally from an H3N2, B-Yamagata, or B-Victoria virus strain [9].
499 Antigenically as well as biochemically such as glycosylation, these virus subtypes and lineages are
500 very different from the H1N1 virus which may impact the imprinting response [55-57]. It is suggested
501 from previous studies such as that from Gostic and colleagues that the subtype of the imprinting
502 virus is essential for subsequent responses to influenza virus infection [14]. Future studies should
503 investigate the responses to split virion vaccination in hosts imprinted with other influenza virus
504 types, subtypes, and lineages. Studies such as these will give insight into which human cohorts will
505 respond to particular vaccine formations. This information will be important for identifying
506 susceptible human populations during virulent influenza seasons and pandemic virus emergence.
507
508 Although great efforts have been made to develop effective influenza virus vaccines, there has
509 been little progress improving vaccine effectiveness as evidenced by the moderate overall
510 effectiveness of the 2018-2019 vaccine at 47% [58]. Several vaccine formulations now exist but it is not
511 known how the preimmune host will response to these platforms. The Centers for Disease Control
512 and Prevention estimated over 80,000 deaths occurred due to influenza virus infection in the 2017-
513 2018 influenza season. Our study takes a step forward to understanding the actual vaccine responses
514 in the majority of humans, since all people over the age of 4 will have an influenza immune history
515 [13]. Together, our findings suggest that H1N1 influenza immune priming leads to more efficacious

516 vaccine responses. Determining how to prime the immune system without the deleterious effects of
517 infection should now be an essential research goal. Importantly, our results may only be extrapolated
518 to human situations with a single previous infection prior to vaccination. Using our strategy, further
519 work is needed to tease out the efficacy and vaccine responses on alternate influenza virus
520 backgrounds such as H3N2 or a layered serial influenza background where sequential infections lead
521 to immunesenescence [59]. As well the responses to vaccination with other vaccine platforms such as
522 the Live Attenuated Influenza Virus (LAIV) vaccine should also be investigated.

523 **5. Conclusions**

524   Although great efforts have been made to develop effective influenza virus vaccines, there has
525 been little progress improving vaccine effectiveness as evidenced by the moderate overall
526 effectiveness of the 2018-2019 vaccine at 47% [58]. Several vaccine formulations now exist but it is not
527 known how the preimmune host will response to these platforms. The Centers for Disease Control
528 and Prevention estimated over 80,000 deaths occurred due to influenza virus infection in the 2017-
529 2018 influenza season. Our study takes a step forward to understanding the actual vaccine responses
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538 the Live Attenuated Influenza Virus (LAIV) vaccine should also be investigated.

539 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1,
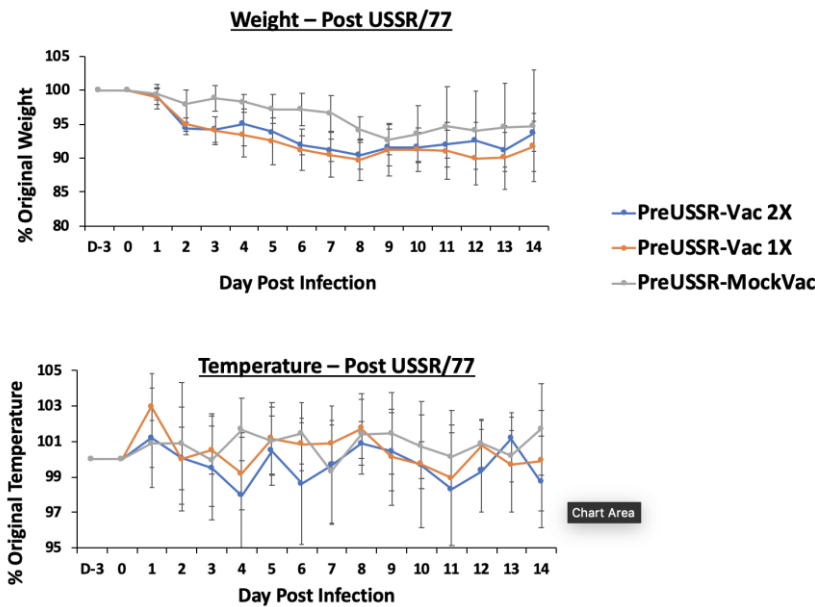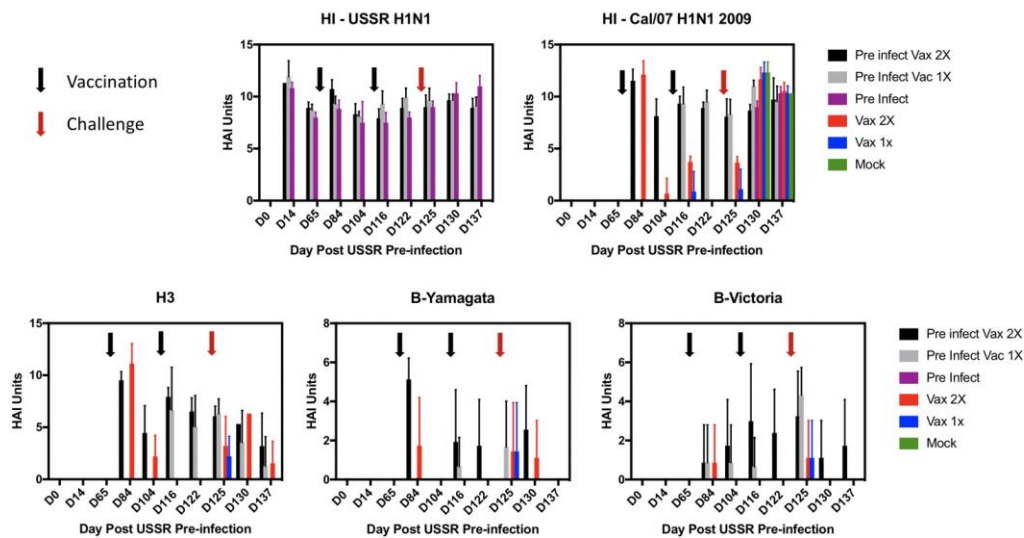

540
541 Figure S1: Weight loss and temperature change following infection with A/USSR/90/1977 to establish a
542 preimmune background.
543 Ferrets were intranasally inoculated with A/USSR/90/1977 in PBS at a dose of 10^6 EID$_{50}$. Ferrets were weighed
544 and temperature was taken daily. The percentage of original values was calculated throughout 14 days.

545

546

Figure S2. Minimal weight loss and temperature change were observed following vaccination with Sanofi FLUZONE® QIV. Both Naïve and preimmune ferrets were vaccinated by intramuscular injection with the Sanofi FLUZONE® QIV split virion vaccine on Day 67 and/or Day 105 following the initial imprinting infection. Ferrets were weighed and temperature was recorded daily. The percentage of original values was calculated and graphed throughout 14 days.
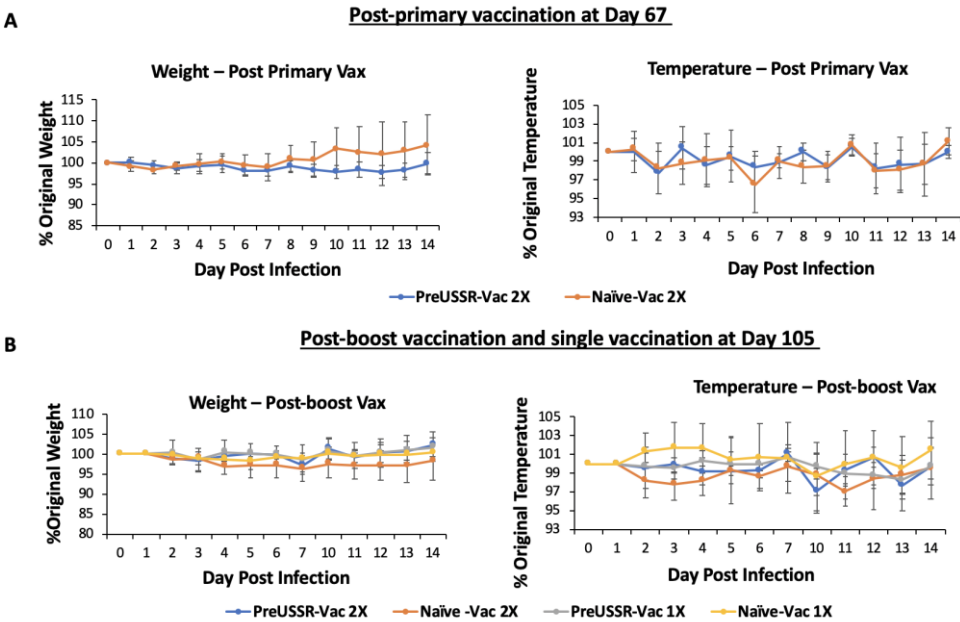
Figure S3: Histogram visualization of HAI titer regulation over the experimental time course showed preimmune ferrets had greater antibody responses compared to naïve-vaccinated ferrets.   The HAI U for each experimental group was calculated at each time point for the HAI titer against the Cal/09 virus. The results were plotted as histograms over the infection time course to visualize the numerical dynamics and changes over time. HAI values are plotted per antigen/virus.   Time of vaccination is indicated by a black arrow.   Time of challenge is indicated by a red arrow.   Day 0 denotes the first day of the study (H1N1 USSR/77 virus inoculation day in appropriate groups).

559

570   **Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the
571   study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to
572   publish the results.

573

574

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