Online Supplemental Material for Powell et al.,

Supplementary Materials and Methods

As stated in the Materials and Methods, animal experiments were monitored for adherence to the ARRIVE guidelines (Kilkenny 2010).

3b. The mouse model is used for these influenza studies because it is inexpensive and convenient and the immunological and pathological manifestations are similar to that seen with human infections of influenza. The studies used give key information about protection that are not possible with in vitro models.

Methods

6a. For most of the experiments we had one control group and two experimental groups such that S-FLU [S-HA(PR8)/N1(PR8)] H1(PR8) was compared to [S-eGFP/N1(PR8)] H1(PR8) with a control VGM (virus growth medium). This gave biological replicates within the experiments. We also used VGM groups compared to [S-HA(PR8)/N1(PR8)] H1(PR8) and [S-eGFP/N1(PR8)] H1(PR8) treated similarly and then challenged at the same time with PR8 or X31 viruses.

b. Animals were put in groups generally having arrived as littermates but for groups where animals were ranged from 6-8 weeks, for instance, these were allocated randomly to groups. Samples for testing of serum antibodies were coded and the experimenter blinded to the identity of each group. Animal groups were not blinded and two different investigators were involved with establishment of clinical scoring criteria. Investigators were aware of different groups and weighing was used to get more objective criteria for euthanasia. Obviously sick animals were scored by an investigator with significant experience of observing infected animals and euthanized for humane reasons. A cut off of weight loss of 20% was used for all experiments as an experimental endpoint.

Scoring was as follows:

Appearance: 0-normal, 1-General lack of grooming, 2-Rough haircoat, 3-Piloerection

Body Condition Score: 0-obese, bulky looking, can’t feel backbone, 0-Fat, can’t feel backbone, 0-Well conditioned can feel backbone if you try, 3-Underconditioned, can feel backbone segment, 4-Emaciated, prominent skeleton, can see hips.

Clinical Signs: 0-Normal colour and movement, 2-Slight changes eg pale paws, 4-Moderate changes eg white paws, 6-Severe changes eg. Bleeding, rear limb paralysis.

Natural Behavior: 0-Normal, 1-Minor changes, 2-Less mobile and alert, isolated, 3-Not alert, very still.

Provoked Behavior: 0-Normal, 1-Minor depression, 2-Moderate changes in expected behavior, 6-Very weak or nearly comatose.

If you have scored >3 more than once add an extra point for each.

Decision Tree:

Total score <5 continue to monitor daily

Total score 6-10. Put wet food on floor, monitor again <12hrs later, euthanize if not better in 12 hours.

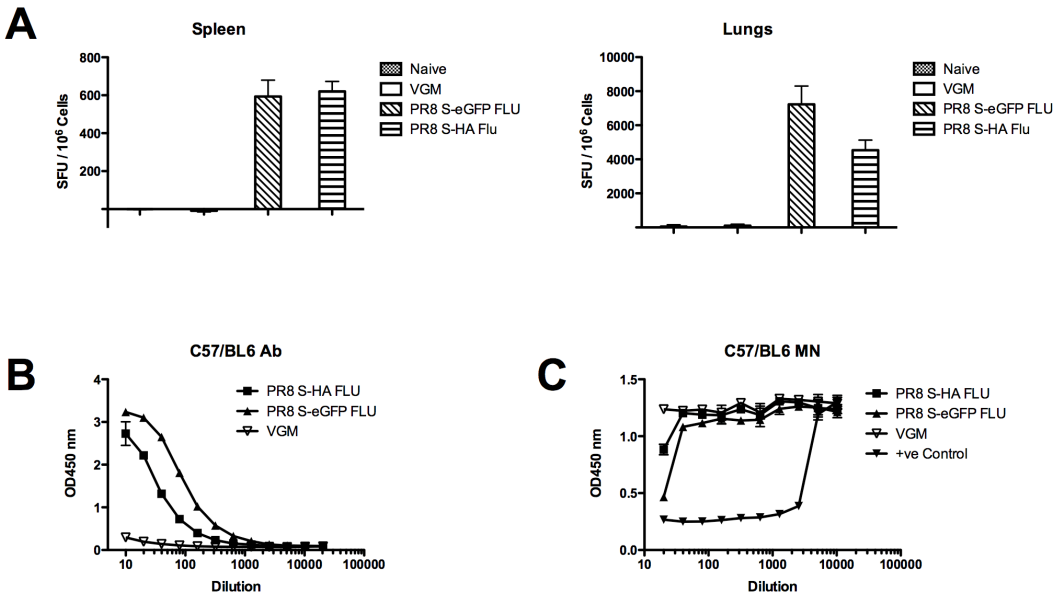
Total score >11 Euthanize immediately.

c. Mice were housed in cages containing six animals. Some were housed in groups of four.
7. Mice were anaesthetised with IsoFlo (Abbott) at a concentration of 3% v/v with an oxygen flow rate of 1500 ml/min. The mice were removed from the chamber and then immunized intranasally with 50 uL of virus suspension or VGM. These procedures were done in a biosafety hood and mice were removed to a clean cage so that recovery could be monitored. Mice were then returned to their home cage. Mice were weighed less than an hour later. Inhalable anaesthetic was considered to be the most appropriate method and this decision was arrived at with consultation from the University Veterinary Services.
8.BALB/c used were either in house derived from Oxford Laboratory Animal Colonies stocks in Blackthorn bred in the BMS for over ten years. Other mice were purchased from Harlan (Bicester), which maintains the BALB/cOlaHsd strain. BALB/cOlaHsd mice originate from the Laboratory Animal Centre, Carshalton UK from the Jackson Laboratory, Bar Harbor, Maine in 1955. In 1976 to Olac (now Harlan Laboratories). C57BL/6 mice were purchased from Harlan (Bicester) strain: C57BL/6JOlaHsd. C57BL/6JOlaHsd mice originate from the Jackson Laboratory, Bar Harbor, Maine. In 1974 to the Laboratory Animal Centre, Carshalton UK. To Olac in 1983 (now Harlan Laboratories).
Six mice per group were generally used such that we would obtain statistically significant information with an unknown effect size. Assuming a mean weight loss of 10% from controls (i.e. 50% of mice approach 20% and have to be euthanized) and 5% from immunized with a standard deviation of 5%, for six mice per group this gives a power of 0.93. Calculated using web based power calculations: http://www.stat.ubc.ca/~rollin/stats/ssize/ accessed 10 Jul 2012. Group sizes were determined from previous experience using mouse models (Powell 2006, Powell 2007).
Animals were housed in an SPF unit within individually vented cages in groups of 4-6. Mice with different treatments were housed in different cages.
14. Mice used were female and weighed between 15-30g dependent on stage of experiment and age of mice at delivery (mice were generally 6-8 wks at commencement of experiments. The range was between 5 and 11 weeks at the beginning of the experiments.
15. For the 4 month experiment shown in Figure 5D there were 72 mice in total in 12 groups of 6. 24 mice were immunized with [SHA(PR8)/N1(PR8)]H1(PR8), 24 with [S-eGFP/N1(PR8)] H1(PR8) and 24 with VGM. 1 mouse from the [S-eGFP/N1(PR8)] H1(PR8) group died during the second round of anaesthesia. 1 mouse from the [S-HA(PR8)/N1(PR8)] H1(PR8) and 1 mouse from the [S-eGFP/N1(PR8)] H1(PR8) groups died 1 month after the second immunization (this meant that the four month challenge groups only had 5 in them in as noted in the figure). 1 mouse in a second [S-HA(PR8)/N1(PR8)] H1(PR8) group was not challenged because it had a large tumor. Therefore there are only 5 mice per group in the 4 month challenge groups although there were six mice initially.
In repeated experiments using both X31 and PR8 challenges we did not see any other unexpected death after immunization. In ten experiments involving a total of 196 animals we only saw three unexpected deaths as documented

above: one was a tumor so is unlikely to be vaccine induced and the two others were a month after the last immunization so unlikely to be a vaccine induced side effect.

References
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Powell TJ, Strutt T, Reome J, Hollenbaugh JA, Roberts AD, Woodland DL, Swain SL, Dutton RW. Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against supralethal challenge with heterosubtypic virus. J Immunol. 2007 Jan 15;178(2):1030-8.
3. Powell TJ, Dwyer DW, Morgan T, Hollenbaugh JA, Dutton RW. The immune system provides a strong response to even a low exposure to virus. Clin Immunol. 2006 Apr;119(1):87-94.

Supplementary Figure 1.



Supplementary Figure 1. C57BL/6 mice were immunised with [S-HA(PR8)/N1(PR8)] H1(PR8) and [S-eGFP/N1(PR8)] H1(PR8) at day 0 and day 14. Twenty days later mice were killed and spleens, lungs and blood collected. Assays were done to determine A: T cell responses by ELISPOT using NP 366-74 from either lung or spleen samples. Data shown are mean +/- SEM for groups of 5 mice individually assayed. B. Antibody levels from pooled serum samples and C. Microneutralisation assay of pooled sera samples from 5 mice.