DNA vaccines encoding antigen targeted to MHC class II induce influenza specific CD8+ T cell responses, enabling faster resolution of influenza disease.

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Conflict of interest statement

The authors declare a potential conflict of interest and state it below

ES, ML and ABF are employed by Vaccibody, which generated the constructs. BB, ABF and GG are inventors on patent applications filed on the vaccine molecules by the TTO offices of the University of Oslo and Oslo University Hospital, according to institutional rules. BB is head of the Scientific panel, and ABF is CSO of Vaccibody AS. They both hold shares in the company.

Author contribution statement

LL, EK, JM, JT performed studies; GG, ES, ML developed and provided reagents; GG, BB, AF, JT wrote paper and JT and AF designed studies.

Keywords

Influenza Vaccines, CD8-Positive T-Lymphocytes, DNA Vaccines, MHC II, Immune targeting

Abstract

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Current influenza vaccines are effective but imperfect, failing to cover against emerging strains of virus and requiring seasonal administration to protect against new strains. A key step to improving influenza vaccines is to improve our understanding of vaccine induced protection. Whilst it is clear that antibodies play a protective role, vaccine induced CD8+ T cells can improve protection. To further explore the role of CD8+ T cells we used a DNA vaccine that encodes antigen dimerised to an immune cell targeting module. Immunising CB6F1 mice with the DNA vaccine in a heterologous prime boost regime with the seasonal protein vaccine improved the resolution of influenza disease compared to protein alone. This improved disease resolution was dependent on CD8+ T cells. However, DNA vaccine regimes that induced CD8+ T cells alone were not protective and did not boost the protection provided by protein. The MHC targeting module used was an anti-I-Ed single chain antibody specific to the BALB/c strain of mice. To test the role of MHC targeting we compared the response between BALB/c, C57BL/6 mice and an F1 cross of the two strains (CB6F1). BALB/c mice were protected, C57BL/6 were not and the F1 had an intermediate phenotype; showing that the targeting of antigen is important in the response. Based on these findings, and in agreement with other studies using different vaccines, we conclude that in addition to antibody, inducing a protective CD8 response is important in future influenza vaccines.

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Ethics statement

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Please detail the consent procedure used for human participants or for animal owners. If not applicable, please state this.

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DNA Vaccines Encoding Antigen Targeted To MHC Class II Induce Influenza Specific CD8$^+$ T Cell Responses, Enabling Faster Resolution Of Influenza Disease.

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Abstract

Current influenza vaccines are effective but imperfect, failing to cover against emerging strains of virus and requiring seasonal administration to protect against new strains. A key step to improving influenza vaccines is to improve our understanding of vaccine induced protection. Whilst it is clear that antibodies play a protective role, vaccine induced $CD8^+$ T cells can improve protection. To further explore the role of $CD8^+$ T cells we used a DNA vaccine that encodes antigen dimerised to an immune cell targeting module. Immunising CB6F1 mice with the DNA vaccine in a heterologous prime boost regime with the seasonal protein vaccine improved the resolution of influenza disease compared to protein alone. This improved disease resolution was dependent on $CD8^+$ T cells. However, DNA vaccine regimes that induced $CD8^+$ T cells alone were not protective and did not boost the protection provided by protein. The MHC targeting module used was an anti-I-E^d single chain antibody specific to the BALB/c strain of mice. To test the role of MHC targeting we compared the response between BALB/c, C57BL/6 mice and an F1 cross of the two strains (CB6F1). BALB/c mice were protected, C57BL/6 were not and the F1 had an intermediate phenotype; showing that the targeting of antigen is important in the response. Based on these findings, and in agreement with other studies using different vaccines, we conclude that in addition to antibody, inducing a protective CD8 response is important in future influenza vaccines.

Introduction

The annual burden of influenza is significant, with the WHO estimating 1 billion cases of infection a year. Of these, an estimate from 2008 suggests that about 90 million cases are in children under 5 years of age (Nair et al., 2011). This huge burden of disease is in spite of there being seasonal vaccines for influenza: these vaccines are not available for the global population and, due to the changing nature of circulating influenza strains, are often not completely effective. Ideally new vaccines with broader cross protection would be developed which address the problem of antigenic drift and the narrow window in which a seasonal vaccine is effective.

For the current generation of strain specific protein vaccines, antibody is a valuable correlate of protection. Currently vaccines are licensed based on a haemagglutination inhibition (HAI) titre of 1:40, a surrogate assay for neutralising antibody. However the HAI assay has limitations, even for assessing antibody, it only measures anti-haemagglutinin responses and does not recognise all haemagglutinin specific antibodies, for example it doesn't detect antibodies that bind the more conserved stem region. Therefore for the next generation of influenza vaccines, particularly for cross-reactive vaccines, better understanding about the relative contributions of different arms of the adaptive immune system in protection is required (Reber and Katz, 2013). For example, HAI titre fails to take into account the role of T cells in the vaccine response to influenza, which may also contribute to cross protection (Altenburg et al., 2015).

Whilst both $CD4^+$ and $CD8^+$ T cells can contribute to protection against influenza, $CD8^+$ T cells are particularly beneficial when they recognise conserved epitopes across multiple influenza strains (Brown and Kelso, 2009). The direct evidence for the role of influenza disease reduction by $CD8^+$ T cells is mostly derived from animal studies, but a recent study correlated influenza specific $CD8^+$ T cells with protection against symptomatic pandemic influenza (Sridhar et al., 2013). Based on their protective role, $CD8^+$ T cells are an attractive target in vaccine development. But, it is of note that $CD8^+$ T cells only function after cellular infection has occurred, acting to limit rather than prevent infection. Understanding the protection provided by vaccine induced $CD8^+$ T cells in the context of virus specific antibody is important in designing new influenza vaccines.

One approach to induce different types of adaptive immune response is to direct antigen to specific antigen presenting cells (Barber, 1997). This can be achieved using dimeric vaccines with targeting modules (either a scFv derived from an antibody, or chemokine) coupled by a dimerization unit to the antigen (Grodeland and Bogen, 2015). Changing the module allows the targeting of different antigen presenting cells, leading to different types of immune responses and enabling the comparison of the relative contribution of different adaptive immune effectors. In previous studies in mice an MHC-targeting module has been shown to induce a dominant IgG1 antibody response with some T cell induction (Grodeland et al., 2013a), whilst an XCL1 chemokine module led to a more skewed $CD8^+$ T cell response (Fossum et al., 2015).

In the current study, we investigated the relative contribution of influenza specific $CD8^+$ T cells induced by a DNA vaccine in a heterologous prime boost regime with a protein vaccine. The protein vaccine induced a strong antibody response, but relatively few CD8 cells. Induction of $CD8^+$ T cells by the dimeric vaccine, improved the resolution of disease, and when CD8 cells were depleted, the enhanced resolution was no longer observed. However, CD8 cells alone were insufficient to protect against infection. Based on this we conclude that vaccine induced CD8 responses are beneficial, but are supplementary to antibody.

Materials and Methods

DNA vaccine constructs

The generation of the DNA vaccine constructs containing the targeting unit, the dimerization unit consisting of h1+h4+C_H3 domains derived from human IgG3, and antigen has been previously described (Fredriksen et al., 2006;Fossum et al., 2015). The constructs either expressed amino acids 18-541, the extracellular domain and part of the transmembrane domain, of influenza A/California/07/2009 (H1N1) haemagglutinin or the conserved IYSTVASSL epitope of H1 (533-541) as the antigen payload and anti-I-E^d MHC class II single chain variable fragment (scFv) from the 14-4-4S monoclonal antibody, that binds the conserved E alpha chain, or murine XCL1 as the targeting unit. All sequences were synthesized by Eurofins MWG (Germany) or GenScript (USA). The synthesized inserts were subcloned into the expression vector pUMVC4a on *Not*I and *Bgl*II, all including either an Ig VH signal peptide or the murine XCL-1 signal peptide to ensure secretion. The αMHCII:HA (Cal/07) construct has been described previously (Grodeland et al., 2013a).

Mouse immunization and infection

6–8 week old female CB6F1, BALB/c or C57BL/6 mice were obtained from Harlan UK Ltd (Bradford, UK) and kept in specific-pathogen-free conditions in accordance with the United Kingdom's Home Office guidelines and all work was approved by the Animal Welfare and Ethical Review Board (AWERB) at Imperial College London. Studies followed the ARRIVE guidelines. Animals were immunised in a prime (d0)-boost (d21)-challenge (d42) regime and culled on day 7 of challenge (d49 relative to prime). For protein immunisation, mice were immunized intramuscularly (i.m.) with 0.1 µg purified surface antigens from influenza strain H1N1 A/California/7/2009 (GSK Vaccines, Siena, Italy) in 50 µl. For DNA vaccination, mice were injected intramuscularly (i.m.) into the *anterior tibialis* with 5 µg plasmid in 50 µl of sterile PBS followed by electroporation (EP). Two lots of 5 pulses of 150V with switched polarity between pulses were delivered using a CUY21 EDIT system (BEX, Japan). For infections**,** mice were anesthetized using isoflurane and infected intranasally (i.n.) with $5x10^4$ PFU of influenza A H1N1 (strain A/England/195/2009). Where used, $CD8^+$ T cells were depleted using two intraperitoneal injections of 0.25 mg anti-murine CD8 antibody clone YTS156 and $CD4^+$ T cells were depleted with 0.125mg each of YTA3 and YTS191 (a kind gift of S. Cobbold, Oxford University) on d-1 and +1 of infection (Tregoning et al., 2013).

Influenza

H1N1 influenza (strain A/England/195/2009), isolated by Public Health England in the UK, April 2009 (Baillie et al., 2012), was grown in Madin-Darby Canine Kidney (MDCK) cells, in serum-free DMEM supplemented with 1 µg/ml trypsin. The virus was harvested 3 days after inoculation and stored at -80°C. Viral titre was determined by plaque assay as previously described (Elleman and Barclay, 2004).

Semi-quantitative antigen-specific ELISA

Antibodies specific to influenza H1N1 were measured using a standardized ELISA (Russell et al., 2016). IgG responses were measured in sera and IgA responses in bronchoalveolar

lavage. MaxiSorp 96-well plates (Nunc) were coated with 1 µg/ml H1N1 surface proteins or a combination of anti-murine lambda and kappa light chain specific antibodies (AbDSerotec, Oxford, UK) and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS. Bound IgG was detected using HRP-conjugated goat anti-mouse IgG (AbD Serotec). Bound IgA was detected using a biotinylated anti-IgA and a streptavidin-HRP. A dilution series of recombinant murine IgG or IgA was used as a standard to quantify specific antibodies. TMB with H_2SO_4 as stop solution was used to detect the response and optical densities read at 450 nm.

Tissue and cell recovery and isolation

Mice were culled using 100 µl intraperitoneal pentobarbitone (20 mg dose, Pentoject, Animalcare Ltd. UK) and tissues collected as previously described (Siggins et al., 2015). Blood was collected from carotid vessels and sera isolated after clotting by centrifugation. Lungs were removed and homogenized by passage through 100 µm cell strainers, then centrifuged at 200 x *g* for 5 minutes. Supernatants were removed and the cell pellet treated with red blood cell lysis buffer (ACK; 0.15 M ammonium chloride, 1 M potassium hydrogen carbonate, and 0.01 mM EDTA, pH 7.2) before centrifugation at 200 x *g* for 5 minutes. The remaining cells were resuspended in RPMI 1640 medium with 10% fetal calf serum, and viable cell numbers determined by trypan blue exclusion.

Influenza viral load

Viral load *in vivo* was assessed by Trizol extraction of RNA from frozen lung tissue disrupted in a TissueLyzer (Qiagen, Manchester, UK). RNA was converted into cDNA and quantitative RT-PCR was carried out using bulk viral RNA, for the influenza M gene and mRNA using 0.1 µM forward primer (5'-AAGACAAGACCAATYCTGTCACCTCT-3'), 0.1 µM reverse primer (5'-TCTACGYTGCAGTCCYCGCT-3') and 0.2 µM probe (5'-FAM-TYACGCTCACCGTGCCCAGTG-TAMRA-3') on a Stratagene Mx3005p (Agilent technologies, Santa Clara, CA, USA). M-specific RNA copy number was determined using an influenza M gene standard plasmid.

Flow cytometry

Live cells were suspended in Fc block (Anti-CD16/32, BD) in PBS-1% BSA and stained with surface antibodies: influenza A H1 $HA_{533-541}$ IYSTVASSL Pentamer R-PE (Proimmune, Oxford, UK), CD3-FITC (BD, Oxford UK), CD4-APC (BD), and CD8-APC Alexa75 (Invitrogen, Paisley, UK). Analysis was performed on an LSRFortessa flow cytometer (BD). FMO controls were used for surface stains.

Statistical Analysis

Calculations as described in figure legends were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Heterologous prime-boost regimes using dimeric DNA vaccines induce both antibody and CD8$^+$ T cell responses and improves resolution of disease.

Vaccine induced, antibody-mediated protection against influenza is well characterised, but CD8$^+$ T cells are also important. DNA vaccines allow the induction of strong cellular responses (Tregoning and Kinnear, 2014) and the use of different targeting modules allow us to compare the relative contributions of different effectors (Grodeland et al., 2015). We compared the response to immunisation using a DNA vaccine encoding dimeric APC-targeted antigen alone or in combination with protein antigens . The DNA vaccine construct for these studies encoded the HA gene from influenza Eng/195 (H1N1) dimerised to an anti-I-E^d MHC class II single chain variable fragment (scFv) with a dimerization unit consisting of h1+h4+C_H3 domains from human IgG3. CB6F1 mice were used for these studies, they are the F1 cross of BALB/c (I-E^d) and C57BL/6 (I-E^b) strains. Mice were immunised once with 5μg DNA encoding the dimeric vaccine construct (αMHCII:HA) intramuscularly with electroporation, with or without a boost (on day 21) using a sub-protective dose of H1N1 proteins (0.1 μg) from CAL/09. Three weeks after the boost immunisation (on day 42), mice were challenged intranasally with H1N1 influenza (strain A/England/195/2009) and culled seven days later (day 49).

Blood was collected prior to infection to determine anti-influenza antibodies. αMHCII:HA primed-protein boosted animals had significantly more antibody than protein alone or αMHCII:HA alone ($p<0.05$, Fig 1A). All immunisations gave some reduction of weight loss following influenza infection. The αMHCII:HA alone group recovered faster on days 6 and 7 after infection than PBS control mice, and a similar phenotype was seen after immunisation with protein alone. However, prime immunisation with DNA then protein boost led to significantly improved recovery from d4 after infection ($p<0.05$ compared to DNA or protein alone on d5 and 6, Fig 1B). After infection, antibody responses in the αMHCII:HA-protein group were the same as the PBS-protein group and levels were 10 fold higher than before infection (Fig 1C). There was some detectable antibody after immunising with αMHCII:HA alone, that was slightly boosted by infection. However, αMHCII:HA alone immunised animals had a significant influenza-specific CD8$^+$ T cell response in the lungs, as measured by pentamer-positive cells, greater than the protein alone or naïve animals ($p<0.05$, Fig 1C). These cells were also induced in the prime-boost group. These data suggest that whilst antibody is protective against influenza infection, antigen specific CD8$^+$ T cells contribute to recovery in the absence or near absence of antibodies.

Accelerated resolution in prime boost regimes is provided by CD8$^+$ T cells

Having observed that heterologous prime-boost immunisation led to faster recovery and the DNA vaccines induced both an influenza specific CD8 and antibody response, we wished to determine the role of the CD8 cells. Mice were immunised with αMHCII:HA with a protein boost or protein alone and responses compared between animals treated with CD8 depleting antibody and control during infection. As seen before, αMHCII:HA-Protein immunisation induced more antibody than protein alone 21 days after the boost immunisation (Fig 2A).

αMHCII:HA-Protein immunised, $CD8^+$-depleted mice lost significantly more weight than the immunised animals with intact $CD8^+$ responses ($p<0.05$ on d6 and 7, Fig 2B). CD8 depletion had no effect on protein-alone immunisation. At day 7 after infection, αMHCII:HA-Protein immunised mice had no detectable viral load and CD8 depletion had no effect on this (Fig 2C). CD8 depletion also had no effect on the antibody response (Fig 2D) or $CD4^+$ T cell number in the lungs (Fig 2E), but led to a significant reduction in both total (Fig 2F) and influenza-specific $CD8^+$ T cells (Fig 2G). From this, we conclude that the improved recovery seen after αMHCII:HA priming before protein vaccination is partially mediated by $CD8^+$ cells.

$CD8^+$ cells in isolation are not sufficient for protection from influenza infection

Since we observed that CD8 cells contribute to the accelerated resolution of disease in the prime boost immunisation, we wished to determine whether vaccines inducing influenza specific CD8 alone could also improve disease resolution. A pilot study was performed to determine the immune response vaccine constructs using different targeting unit/ antigen combinations, in order to select the ones that gave the greatest $CD8^+$ T cell responses. Mice were immunised with constructs encoding either anti-I-E^d scFv or the XCL1-targeting module with either the full HA surface domain (of Cal/07) or the K^d immunodominant epitope alone in H1 haemagglutinin ($HA_{533-541}$ IYSTVASSL). The groups immunised with constructs encoding the epitope alone were not protected against influenza infection (Fig 3A, B). The more complete HA constructs offered modest protection, with αMHCII:HA immunised animals recovering slightly faster than the naïve animals and the XCL1:HA immunised animals gaining weight on d7 post infection. There were striking differences in the antibody responses: only animals immunised with a construct expressing the whole HA had detectable antibody responses, and the response to the MHCII-targeting construct was greater than the XCL1 (Fig 3C). Whilst the antibody responses were poor to these constructs, there was substantial recruitment of influenza-specific $CD8^+$ T cells. All immunised groups had influenza-specific T cells in the lungs, but there were greater responses in the epitope-immunised animals (Fig 3D). From this pilot study, we conclude that the epitope only vaccines induce a stronger CD8 response.

To assess the relative contributions of $CD8^+$ cells versus antibody, we took advantage of the differential responses to the αMHCII:HA or αMHCII:Epitope constructs, with either a DNA or protein boost, prior to infection with influenza. Prime-boost regimes with protein or αMHCII:HA (Eng/195) led to significant protection against infection, with little difference between the homologous or heterologous prime-boost regimes in weight loss (Fig 4A). Protein containing regimes (Protein-Protein or αMHCII:HA-Protein) had slightly less detectable viral RNA in the lungs, than the αMHCII:HA homologous regime (Fig 4B). The groups receiving a protein vaccination had more antibody than the other groups (Fig 4C), though it was surprising that there was no boost in antibody response after the second protein immunisation. The regimes using the αMHCII:Epitope induced the greatest level of $CD8^+$ cells in the lungs after infection ($p<0.05$, Fig 4D), but the αMHCII:Epitope immunised animals were not protected against infection, losing a similar amount of weight as naïve animals and having an equivalent viral load. Priming with αMHCII:Epitope followed by

protein did lead to significantly more $CD8^+$ T cells than Protein-Protein, but had little effect on protection. As seen before, the protein-only immunisation regime did not induce any influenza-specific $CD8^+$ T cells. These data suggest influenza specific $CD8^+$T cells targeting the IYSTVASSL epitope of H1 are not sufficient to protect against infection.

Mouse strain key determinant of protection for MHCII targeting constructs

In previous studies using similar DNA vaccine constructs in BALB/c mice, complete protection against Cal/07 infection was observed after a single DNA vaccination(Grodeland et al., 2013a). Possible sources of differences include the amount of DNA delivered (25μg in published, 5μg in current), the route of delivery (i.d. in published, i.m. in current), viruses used for challenge (Cal/07 in published, Eng/195 in current: the HA genes from Cal/07 and Eng/195 are 99% identical, with 4 amino acid changes), the mouse strains used (BALB/c in published, CB6F1 in current), or the antigens inserted into the MHCII-targeted construct. To ensure there was no difference between constructs used in the current study and the published constructs, we compared immunisation with the construct used in the previous study (Grodeland et al., 2013a) and a construct expressing the HA from Eng/195. CB6F1 mice were immunised with 5 μg of each construct with electroporation, and 28 days later they were infected intranasally with $5x10^4$ PFU of ENG195. Weight was measured daily after infection, there was no difference between mice immunised with the two vaccine constructs; immunised mice recovered faster than naïve mice on d7 after infection (Fig 5A). Significantly more viral RNA was detected in the lungs of previously naïve animals than in immunised animals, and there was no difference in viral load between mice immunised with either construct (Fig 5B). Both constructs induced an immune response, as there was detectable specific IgG in the sera at d7 (Fig 5C) and flu-specific $CD8^+$ T cells in the lung (Fig 5D) in immunised but not naïve animals. From this, we conclude that the incomplete protection observed in the initial studies was not due to the construct, the antigen targeted, or the challenge virus, suggesting that mouse strain may be important, though the dose and route may also contribute to differences seen.

Compatibility of host strain and vaccine construct MHC-targeting unit is critical in level of protection

The targeting unit of the MHC vaccine construct is based on an scFv, derived from the 14-4-4S monoclonal antibody that binds the conserved E alpha chain of the $I\text{-}E^d$ MHCII molecule, which is expressed in mouse strains that are $H\text{-}2^d$. We have previously observed that mouse strain is critical in the recall immune response to respiratory viral infection (Tregoning et al., 2010). Previously published studies with similar MHC targeting vaccine constructs used BALB/c mice ($H\text{-}2^d$) and the current studies used CB6F1 mice, which are mixed $H\text{-}2^d$ and $H\text{-}2^b$. To test whether mouse strain has an effect on the immune response to the vaccine, we immunised BALB/c ($H\text{-}2^d$), C57BL/6 ($H\text{-}2^b$) and CB6F1 (mixed $H\text{-}2^d$ and $H\text{-}2^b$) with the αMHCII:HA construct. These animals were then challenged with influenza. Naïve animals started losing weight on day 2 after infection and this weight loss continued to day 7, at which point the animals were culled (Fig 6A). There was no significant difference in the magnitude or the profile of the weight loss between the naïve animals regardless of strain, indicating that baseline susceptibility to influenza was similar. However, there was a striking

difference in protection based on MHC genotype. BALB/c were more protected than F1 mice, which were more protected the C57BL/6 mice, directly reflecting the amount of I-E^d MHC (Fig 6B-D) and reflecting the previously published study (Grodeland et al., 2013a). Likewise, there was only a reduction in viral load in the BALB/c and F1 immunised mice (Fig 6E). There was detectable influenza-specific antibody (Fig 6F) and $CD8^+$ T cells (Fig 6G) in both the BALB/c and CB6F1 mice and there was no difference between the two strains, suggesting that there are other components that contribute to protection against infection. The BALB/c mice had a higher proportion of $CD4^+$ T cells in the lungs, which may have contributed to protection (Fig 6H), but in a separate study when treated with CD4 depleting antibody during challenge, there was no effect on resolution of disease after depletion (Fig 6I).

In the CB6F1 mice, the regimes that induced $CD8^+$ T cells alone did not protect against infection. Since we observed a difference between BALB/c and CB6F1 mice in protection following immunisation with the αMHCII:HA construct, we wished to determine whether there was a difference in the protective capacity of the $CD8^+$ T cells induced in H-2^b mice. BALB/c were immunised twice with the MHCII-epitope construct prior to infection with influenza. Mice were not protected against infection (Fig 6J) despite inducing an extremely high influenza-specific CD8 response (Fig 6K). As with the CB6F1 mice, no antibody response was seen after immunisation with this construct (data not shown). These studies clearly demonstrate the effect of the targeting module on the response.

Discussion

In this study we observed that a DNA vaccine encoding a dimeric construct that targets haemagglutinin to antigen presenting cells can induce an influenza specific $CD8^+$ T cell response, which in the context of antibody can lead to more rapid recovery from infection. CD8 cell depletion removed the extra protection provided by the DNA vaccination. It should be noted that the MHCII:HA DNA-prime protein-boost regime induced more antibody than protein alone prior to infection which will contribute to the additional protection seen; but the depletion studies suggest that the additional protection provided by elevated antibody was secondary to that provided by CD8. From this we conclude that CD8 contribute to protection against influenza infection, but are insufficient when acting alone.

The H1 haemagglutinin epitope (IYSTVASSL) only DNA constructs were insufficient to protect against influenza infection in spite of inducing robust CD8 responses in the lung during infection. There were a number of possible reasons why immunisation that only induces a CD8 response fails to protect against influenza challenge including immunopathology, the infectious dose used, the DNA vaccine dose used, targeting a poorly protective epitope or immunising the wrong tissue. Excess $CD8^+$ T cells can be associated with disease, both for influenza (Enelow et al., 1998) and respiratory syncytial virus (RSV) infection (Tregoning et al., 2008). But there was little evidence for CD8 cells causing enhanced immunopathology in the current study – for example the MHCII:Epitope construct induced little antibody and high levels of CD8 cells, but the disease profile was the same as naïve mice. A different epitope might be more protective, the current study evaluated a CD8 epitope in haemagglutinin, CD8 responses against the NP protein of influenza have been explored for vaccine candidates (Baranowska et al., 2015). Though in other studies, the IYSTVASSL (HA533) epitope has been used as a heterologous boost vaccine, expressed by *Listeria* (Pham et al., 2010), leading to heterosubtypic immunity; differences in route, dose, regime and vector of delivery could all contribute to the differences seen. It is possible that in the absence of antibody, CD8 cells are being swamped by virus: with a smaller infectious challenge dose, CD8 cells may have provided more protection (Moskophidis and Kioussis, 1998), but the viral dose used has been carefully titrated to give a clear disease phenotype. It was of note that the αMHCII:HA and other DNA vaccines used in other studies (Walters et al., 2014;Kinnear et al., 2015) were protective against the same dose of the same virus; notably all of these regimes induced antibody and CD8 T cells, indicating that both are required.

Another possibility is that the CD8 cells induced by vaccination were in the wrong tissue. Systemic vaccination is most likely to lead to systemic T cell memory, resident in the spleen, whereas CD8 in the lung are required to clear the infection. The time taken to recruit cells in response to infection from the systemic to the local compartment may account for the failure to clear the infection. Tissue resident memory CD8 cells have been shown to be critical in protection and vaccine regimes that induce them have a significantly improved outcome compared to systemic vaccination (Wakim et al., 2015). One approach might be to use live viral vaccines, the use of the live attenuated influenza vaccine led to the induction of influenza specific CD8 T cells in the lungs (Chen et al., 2011), we have recently shown that

heterologous prime boost strategies including viral vectors can alter the immune outcome (Badamchi-Zadeh et al., 2016).

One of the striking observations was the effect of mouse MHC genotype on the response to the MHC targeting vaccine constructs. This shows the targeting component of the dimeric vaccine is critical in the response it induces. But the homozygous I-E^d strain (BALB/c) were most protected against infection, in spite of having broadly similar antibody and $CD8^+$ T cell responses to the heterozygous CB6F1 mice, C57BL/6 mice were not protected due to their lack of I-E^d. One possibility is that haemagglutinin specific CD4 cells were also induced by the vaccine and there more of these in the BALB/c mice than the CB6F1. There are 2 well characterised MHCII epitopes in H1 derived haemagglutinin SVSSFERFEIFPK (H2-IE^d positions 124-136) and HNTNGVTAACSHE (H2-IA^d positions 139-151) and αMHCII:HA can induce responses against these (Grodeland et al., 2013a). The role of CD4 cells in protection against influenza is less well characterised than CD8, but recent studies have shown a correlation between CD4 T cell responses and protection in a human influenza challenge study (Wilkinson et al., 2012). Whether these cells play a role and what role they play – either as helpers (Nakanishi et al., 2009) or as cytotoxic T cells (van de Berg et al., 2008) is not clear, though the depletion of CD4 during challenge had no effect on disease outcome, suggesting they are not acting as cytotoxic effectors, but may be important in priming the response.

The best protection was observed when both CD8 and antibody were induced. This reflects other studies using the dimeric vaccine constructs expressing haemagglutinin (Grodeland et al., 2013a;Grodeland et al., 2013b;Fossum et al., 2015) all of which induced both antibody and CD8 T cells. We believe that CD8 play a critical role in the later stages of the infection leading to viral clearance and recovery from influenza infection; providing an adjunct to antibody mediated protection. Studies in human RSV showed that the probability of protection from antibody follows a sigmoidal distribution suggesting a role for other factors (Habibi et al., 2015). We propose a model where antibody prevents the initial colonisation, but if antibody is evaded by the virus then CD8 cells enable more rapid clearance. In this context, vaccines that can induce local CD8 responses may be of value, particularly if they target conserved epitopes.

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Author Contribution

LL, EK, JM, JT performed studies; GG, ES, ML developed and provided reagents; GG, BB, AF, JT wrote paper and JT and AF designed studies.

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Figure Legends

Figure 1. Heterologous prime-boost regimes using dimeric DNA vaccines induce both antibody and CD8$^+$ T cell responses and improves resolution of disease.

Mice were immunised intramuscularly (i.m.), immediately followed by electroporation, with 5 μg DNA encoding an MHCII-targeting:HA construct or 0.1 μg H1N1 proteins or DNA then protein. Animals were infected intranasally (i.n.) with $5x10^4$ PFU A/England/195/2009 H1N1 influenza. One day before infection IgG was assessed in sera (A). Weight change was measured after infection (B). H1 Influenza specific IgG by ELISA (C) and influenza-specific CD8$^+$ T cells (D) were measured on day 7 after infection. Lines and points represent mean of n≥4 mice **p<0.01, ***p<0.001 between MHCII:HA-Protein and MHCII:HA, # p<0.05 between MHCII:HA and Protein alone measured by one way (B, C) or two-way ANOVA (A).

Figure 2. CD8$^+$ T cells required for accelerated resolution of DNA prime boost regimes.

CB6F1 mice were immunised i.m. with 5 μg DNA encoding MHCII:HA then 0.1 μg H1N1 proteins, or protein alone. 21 days later mice were infected intranasally with H1N1 influenza. CD8$^+$ T cells were depleted by antibody (YTS156) on d-1 and +1 of infection. One day

before infection IgG was assessed in sera (A). Weight change was measured after infection (B). M gene copy number (C), H1 Influenza specific antibody by ELISA (D), CD4+ (E) and CD8+ (F) and influenza specific $CD8^+$ T cells (G) were measured on day 7 after infection. Lines and points represent mean of n=5 mice *p<0.05, **p<0.01 between MHCII:HA-Protein and MHCII:HA-Protein αCD8 measured by one way ANOVA.

Figure 3. Pilot study comparing CD8 response to different dimeric vaccine constructs. CB6F1 mice were primed i.m. with 5 μg DNA constructs targeting either MHC II or the XCR1 chemokine receptor, conjugated to the full HA peptide or the immunodominant epitope alone (epi). Animals were infected i.n. with Eng/195 H1N1 influenza. Weight change was measured after infection (A), with day 7 weight alone shown for clarity (B). H1 Influenza specific antibody by ELISA (C) and influenza specific CD8 T cells (D) were measured on day 7 after infection. Lines and points represent mean of n≥3 mice.

Figure 4. The combination of antigens used in the prime boost regime is critical in determining outcome. CB6F1 mice were primed i.m. with 5 μg DNA constructs encoding MHCII:HA or MHCII:epitope, or 0.1 μg HA1 protein prior either heterologous or homologous boost. 21 days after boost, animals were infected i.n. with Eng/195 H1N1 influenza. Weight change was measured after infection (A). M gene copy number (B), H1 Influenza specific antibody by ELISA (C) and influenza-specific $CD8^+$ T cells (D) were measured on day 7 after infection. Lines and points represent mean of n=5 mice *p<0.05, **p<0.01 measured by one way ANOVA .

Figure 5. Incomplete protection with both Cal07- and Eng195-encoding constructs in CB6F1 mice. Mice were immunised intramuscularly with 5 μg DNA encoding different antigen-targeting module constructs with electroporation, prior to infection intranasally with $5x10^4$ PFU A/England/195/2009 H1N1 influenza. Weight change was measured after infection (A). M gene copy number (B), H1 Influenza specific antibody by ELISA (C), and influenza-specific $CD8^+$ T cells (D) were measured on day 7 after infection. n=5 animals per group, ** p<0.01 and *** p<0.001 using ANOVA and post test.

Figure 6. Strain compatibility with MHC-targeting module affects protection against challenge. Mice were immunised intramuscularly with 5 μg DNA encoding an $I\text{-}E^d$ MHCII targeting-HA construct with electroporation, prior to infection intranasally with $5x10^4$ PFU A/England/195/2009 H1N1 influenza. Weight change was measured after infection (A). The same data is presented by strain for clarity: BALB/c (B), CB6F1 (C) and C57BL/6 (D). M gene copy number (E), H1 Influenza specific antibody by ELISA (F), influenza-specific $CD8^+$ T cells (G) and % $CD4^+$ T cells (H) were measured on day 7 after infection. Weight loss of MHCII:HA immunised BALB/c mice treated with CD4 depleting antibody during infection (I). BALB/c mice were immunised two times intramuscularly with 5 μg DNA encoding an $I\text{-}E^d$ MHCII targeting-epitope construct with electroporation prior to infection. Weight change was measured after infection (J). On day 7 after infection, percentages of influenza-specific $CD8^+$ T cells were quantified (K). n=5 animals per group.

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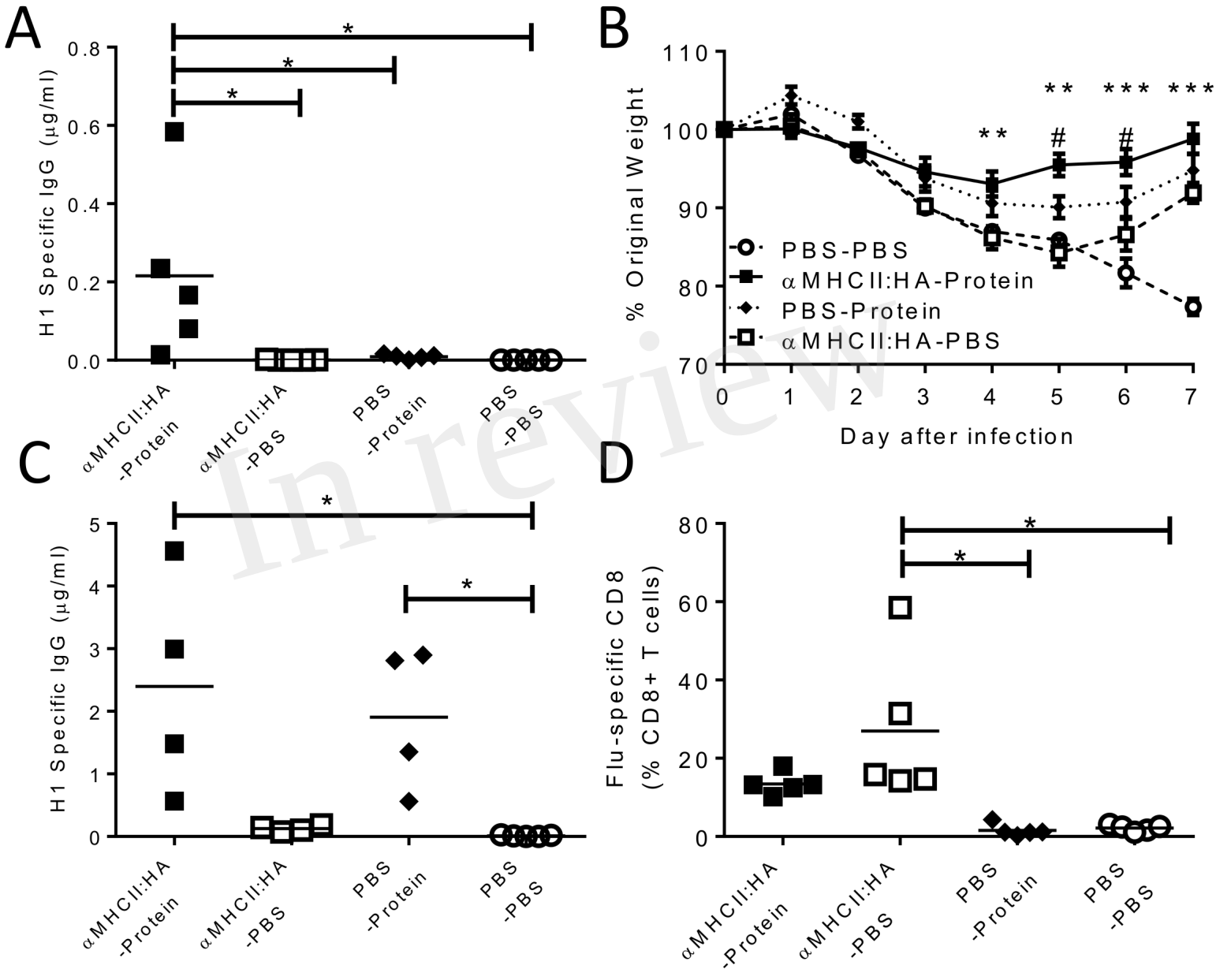

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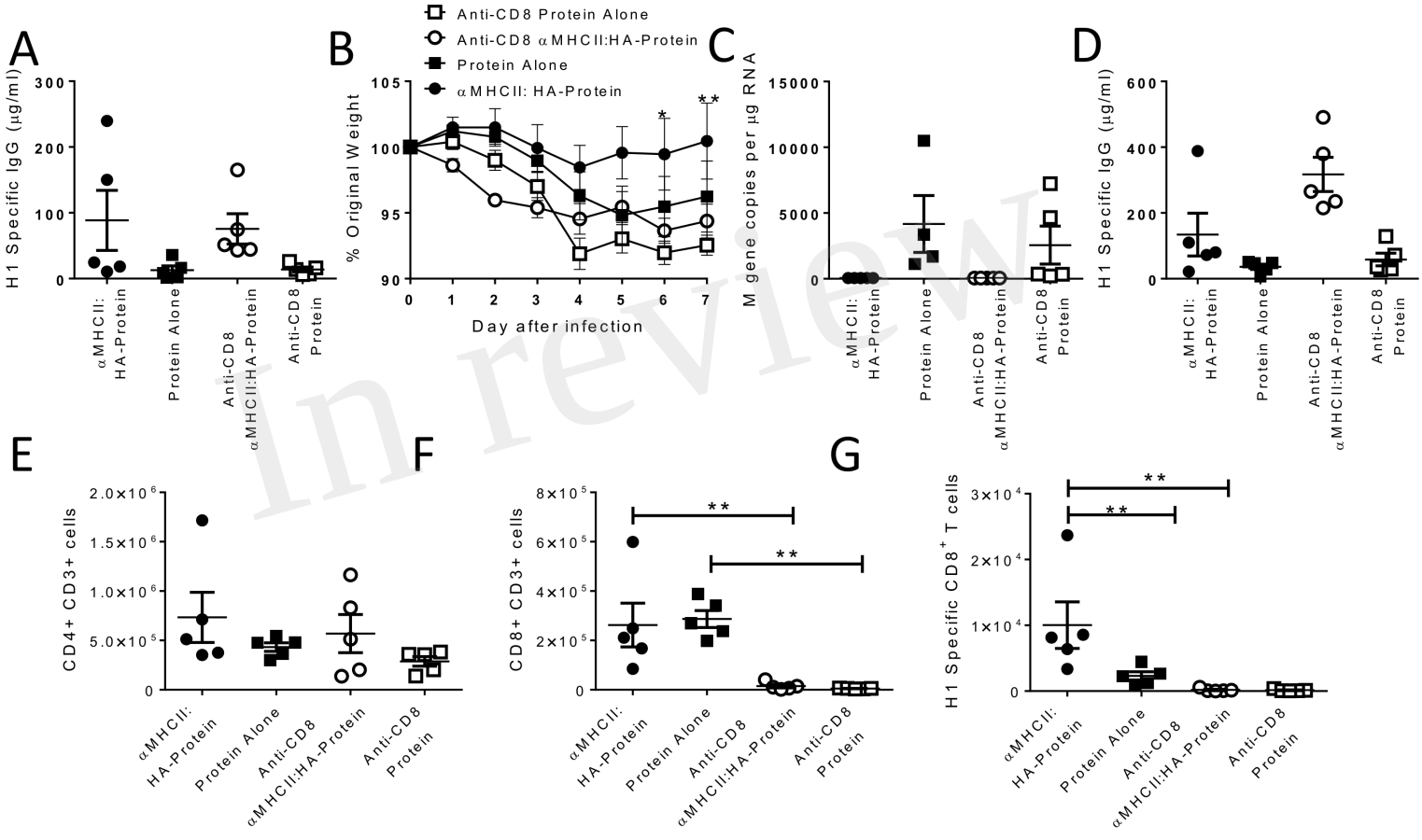

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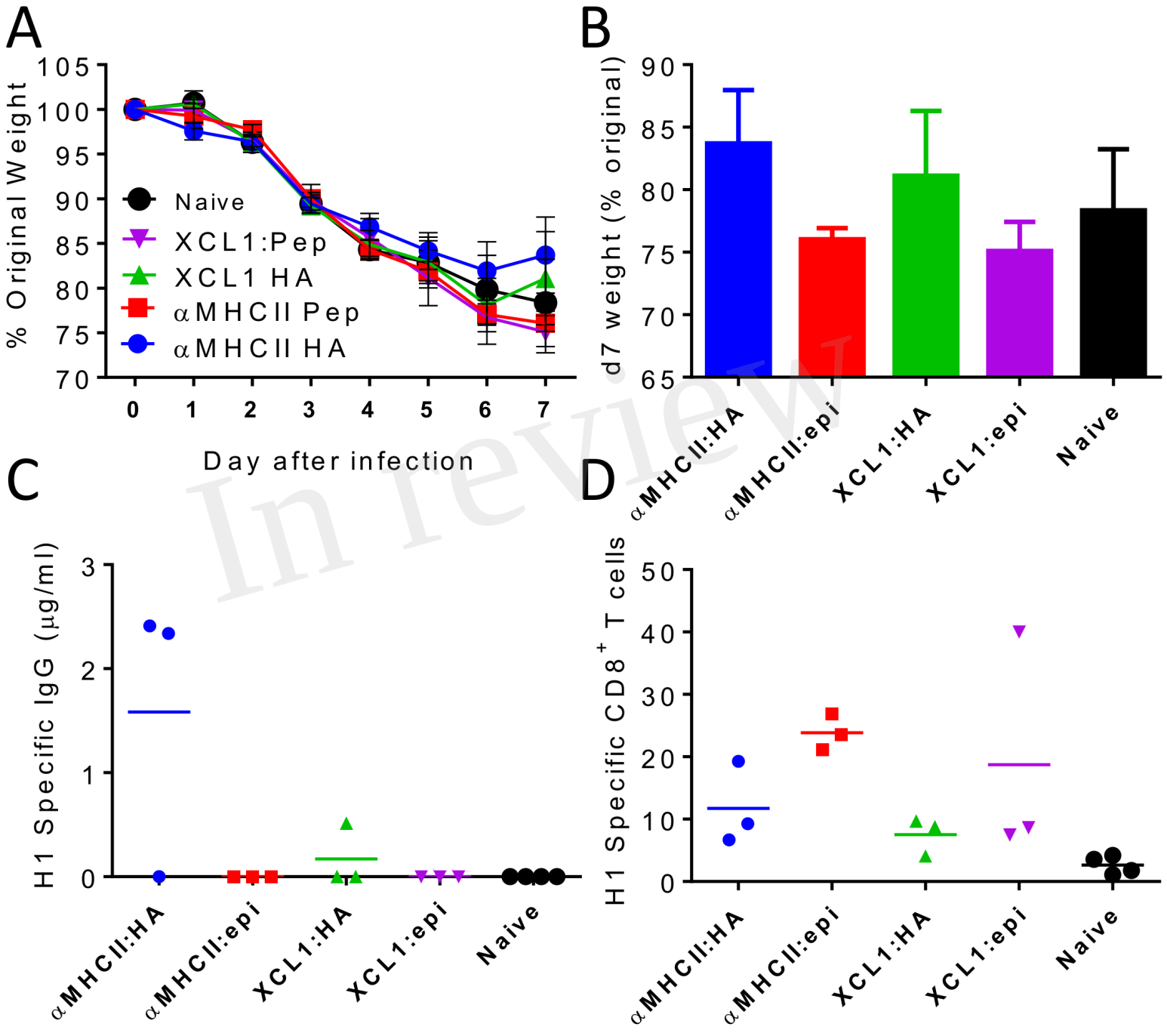

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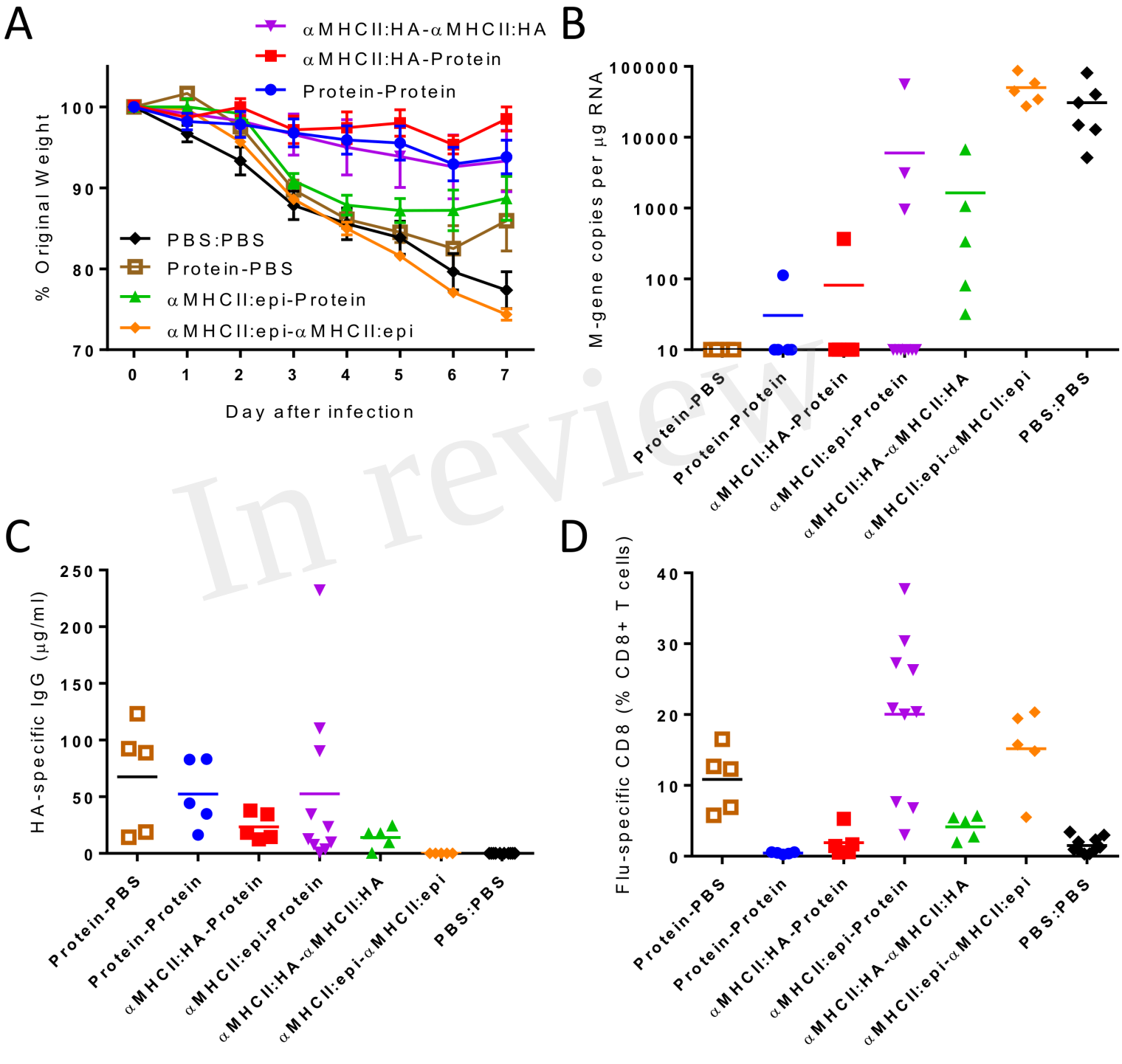

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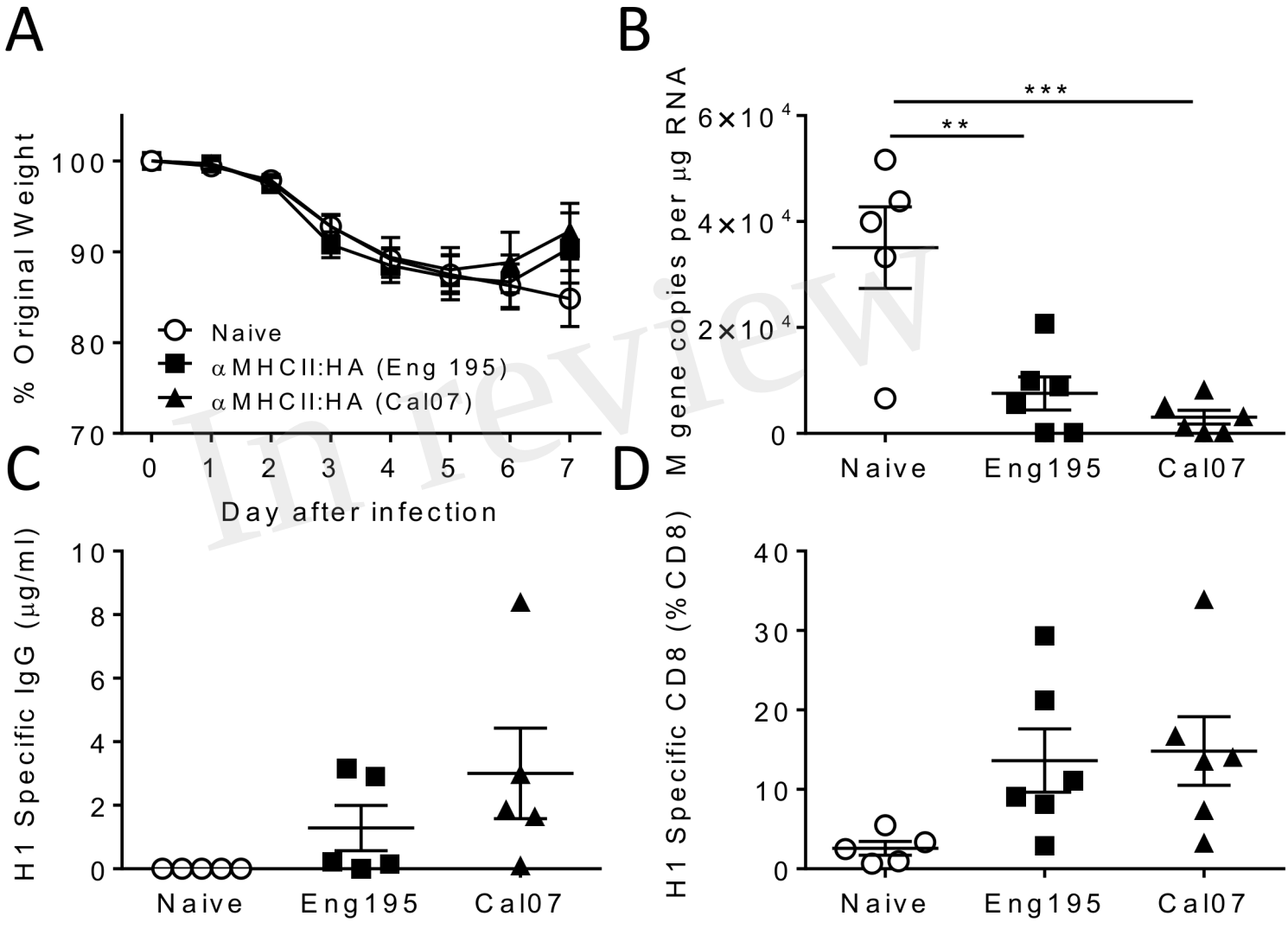

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