Vaccine 37 (2019) 2624–2633

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Targeting M2e to DEC-205 induces an enhanced serum antibody-dependent heterosubtypic protection against influenza A virus infection

H.O. Padilla-Quirarte [a,b], O. Badillo-Godinez [d], L. Gutierrez-Xicotencatl [d], Y. Acevedo-Betancur [a], J.D. Luna-Andon [a], J.L. Montiel-Hernandez [e], D.V. Lopez-Guerrero [c,1], F. Esquivel-Guadarrama [a,*,1]

[a] LIV, Facultad de Medicina, UAEM, Cuernavaca, Morelos, Mexico
[b] Instituto de Biotecnología, UNAM, Cuernavaca, Morelos, Mexico
[c] Facultad de Nutrición, UAEM, Cuernavaca, Morelos, Mexico
[d] CISEI-INSP, Cuernavaca, Morelos, Mexico
[e] Facultad de Farmacia, UAEM, Cuernavaca, Morelos, Mexico

ARTICLE INFO

Article history:
Received 6 November 2018
Received in revised form 23 January 2019
Accepted 15 February 2019
Available online 5 April 2019

Keywords:
Influenza
M2e
DEC-205
Dendritic cells
Heterosubtypic vaccine

ABSTRACT

The ectodomain of the influenza A virus (IAV) M2 protein (M2e) is highly conserved, and it represents a promising candidate for the development of an "universal vaccine". However, the low immunogenicity associated to M2e in a natural infection or in response to seasonal vaccines has led to explore new approaches to enhance it. In recent years, it has become clear that targeting antigens to dendritic cells (DC) is an efficient way to enhance immune responses against pathogens. In this work, the M2e peptide was chemically cross-linked to a monoclonal antibody (mAb) specific for DEC-205 (α-DEC-205:M2e), present on DC. BALB/c mice were inoculated subcutaneously (s.c.) three times with the conjugate equivalent to 1 μg of M2e, in the presence of polyinosinic-polycytidylic acid (poly I:C) as adjuvant. As controls, other groups of mice were inoculated under the same conditions with M2e cross-linked to an isotype control mAb (isotype:M2e), 5 μg of free M2e peptide, ovalbumin (OVA) cross-linked to the α-DEC-205 mAb (α-DEC-205:OVA) or poly I:C alone. Immunization with α-DEC-205-M2e induced high levels of serum antibodies (Abs) compared to isotype:M2e or to free M2e peptide, and in all cases IgG1 was predominant over IgG2a Abs. Furthermore, immunization with the α-DEC-205:M2e conjugate did not prevent morbidity, but it induced up to 76% protection against a heterosubtypic IAV lethal challenge. Contrasting with the 20 to 40% protection induced by isotype:M2e or by free M2e peptide. The protection induced by α-DEC-205:M2e conjugate was dependent on non-neutralizing serum Abs and independent of effector $CD4^+$ T cells. These results show that targeting M2e to DEC-205 is a very effective alternative to induce strong heterosubtypic protection against an IAV infection.

1. Introduction

Influenza A viruses (IAV) are still a continuous threat to public health. Every year, up to half a million deaths are caused by seasonal strains, and there is always a potential risk that a new strain emerges, causing a pandemic. Even though there are seasonal vaccines, they must be reformulated annually, and their efficacy depends on a good prediction of circulating strains every season. On the other hand, immunity against pandemics could be nonexistent or poor, depending on the characteristics of the new strain [1].

The idea of a "universal vaccine." which could induce protection against all subtypes of IAV, has been raised by many research groups around the world. The ectodomain of matrix-2 protein (M2e), which is highly conserved among IAV (particularly among viruses with the same host restriction), has been proposed as an important target antigen to this effect [2,3].

The M2 protein of IAV is a channel-shaped homotetrameric type III protein, which traverses the virion from outside to inside. This protein has three domains: a C-terminal cytoplasmic domain (54 aa), a transmembrane domain (19 aa), and a short N-terminal domain of M2e ($M2_{1-24}$) located on the outside of the virion (ectodomain), and has > 90% aa identity among influenza isolates [4]. M2 plays an active role during the entry phase of IAV, allowing the entrance of protons to the virion core and causing the dissociation of viral ribonucleoproteins (vRNPs) from capsid protein M1

* Corresponding author at: Laboratorio de Inmunología Viral, Facultad de Medicina, Universidad Autónoma del Estado de Morelos, Calle Iztaccihuatl, Esq. Leñeros, Col. Volcanes Cuernavaca, Morelos C.P. 63250, México.
E-mail address: fernando.esquivel@uaem.mx (F. Esquivel-Guadarrama).
[1] These authors contributed equally.

https://doi.org/10.1016/j.vaccine.2019.02.050
0264-410X/

and their subsequent release into the cytoplasm [5]. Moreover, M2e balances the pH in the *trans*-Golgi network (TGN), thus preventing an early hemagglutinin (HA) membrane fusion conformation [6].

Experimental data have revealed that M2e can induce protective heterosubtypic immune responses. Even though M2e-specific-Abs are non-neutralizing, they can help eliminate infected cells by recognizing M2e on their surface. They engage macrophages or NK cells via Fcγ receptors, promoting phagocytosis and/or antibody-dependent cell cytotoxicity (ADCC) [7–9]. Furthermore, several studies have reported specific T cell epitopes (CD4 and CD8) within M2e, which may contribute to the protection [10–13].

However, immunogenicity to M2e is very poor within the course of a natural IAV infection, and specific Abs are inefficient in recognizing the few molecules present on the virion membrane (about 16), due to the steric hindrance exerted by major membrane glycoproteins [HA and neuraminidase (NA)] [14]. To overcome this, M2e-based vaccines have been tested in different animal models (mice, ferrets, and rhesus monkeys). Different approaches have also been tried to increase its immunogenicity, like coupling it to carrier proteins [2], including it in virus-like particles (VLPs) [15], codifying it in DNA vaccines [16], and using a variety of adjuvants. In general, these vaccines have been shown to induce high levels of heterosubtypic protection.

In this context, targeting antigens to antigen-presenting cells (APC), and especially to DC, is a strategy that could be used to boost cellular and humoral immunity to M2e. On their surface, DC are equipped with endocytic receptors, which promote antigen internalization and the subsequent processing and presentation to T cells. These features have been used for designing experimental vaccines that potentiate immunogenicity against particular antigens, when they are linked to receptor-specific mAbs, thus optimizing their delivery to DC [17].

Among these receptors, DEC-205 is particularly attractive for DC-based vaccines. DEC-205 is a C-type lectin predominantly expressed on the surface of skin DC and with a minor expression reported on B cells, NK cells, T cells and plasmacytoid DC (pDC) [18,19]. It appears that the main biologic role of DEC-205 is to promote tolerance by sensing apoptotic cells and the subsequent presentation of self-antigens to T cells [20,21]. However, when the antigen is targeted to this receptor coupled to mAbs in the presence of adjuvant, the delivery and the potential presentation of DC to CD4 T cells (via MHC-II) or the cross-presentation to CD8 cells (via MHC I) are greatly enhanced, with minimal quantities of antigen compared to a non-targeted antigen. Furthermore, evidence suggests that targeting antigens to DEC-205 through parenteral immunization leads to strong and protective T-cell mucosal responses, which is particularly relevant for the design of vaccines against viruses that infect mucosa [21,22]. Moreover, under experimental conditions, activated B cells are capable of presenting antigen to T cells via DEC-205 as efficiently as DC. On the other hand, germinal center (GC) B cells targeted via DEC-205 increase their potential to interact with follicular helper T cells (Tfh), and they generate plasma cells or reenter the dark zone of the GC for further proliferation and maturation [23,24].

The aim of this work was to investigate whether targeting the M2e peptide, which contains epitopes of both B and T cells, to DC via DEC-205 could improve its immunogenicity compared to M2e-free peptide. We found that M2e targeted to DEC-205, in the presence of adjuvant, greatly enhanced the heterosubtypic protection against an IAV infection compared to M2e-free peptide or M2e peptide conjugated to an isotype control mAb. We also found that this protection was dependent on non-neutralizing serum antibodies but independent of effector $CD4^+$ T cells.

2. Material and methods

2.1. Mice

Specific-pathogen-free, 4 to 8 weeks old female BALB/c mice were provided by the Biotechnology Institute (IBT, UNAM, Cuernavaca, Morelos, Mexico). For experimental procedures, mice were housed either in the IBT or in the Faculty of Medicine (UAEM, Cuernavaca, Morelos, Mexico) under standard light/dark cycle (12 h/12 h) and provided with food and water *ad libitum*.

2.2. Cells

Madin-Darby canine kidney cells (MDCK; ATCC CCL-34) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL penicillin/100 μg/mL streptomycin (GIBCO). The hybridomas NLDC145 (rat anti-mouse DEC-205, IgG2a), III-10 (control of rat isotype, IgG2a), GK1.5 (rat anti-mouse CD4, IgG2b) (ATCC TIB-207) and M2-1C6-4R3 (mouse anti-M1 of influenza, IgG1; ATCC HB64) were maintained in RPMI medium, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 20 μg/mL gentamycin (GIBCO). The hybridomas NLDC145 and III-10 were kindly donated by Ralph Steinman's group (Laboratory of Cellular Physiology, The Rockefeller University, New York) [25]. DCs were derived from 9 to 12 days cultures of mouse bone marrow cells in the presence of GM-CSF (Invitrogen), as previously described [22].

2.3. Viruses

Influenza virus A/H1N1/New Caledonia/20/99 and A/H3N2/NT/60/68 were kindly donated by Carlos Arias (IBT, UNAM, Cuernavaca, Morelos, Mexico) and Jack Bennink and Jonathan Yewdell (Laboratory of Viral Immunology, NIAID, NIH, Bethesda, Maryland), respectively. Viruses were expanded *in vitro* in MDCK cells, or in 10 days old chicken embryos (Alpes, Mexico) infected and incubated for 48 h at 34 °C. For *in vivo* infections, viruses were adapted to mouse by serial passages (14 times) in BALB/c mice as previously described [26,27]. Cell lysates, allantoic fluid, and lung homogenates were aliquoted and stored at −80 °C until their use.

2.4. Peptides and antibodies

Peptides derived from a consensus human virus sequence of M2e (aa 1-24; MSLLTEVETPIRNEWGCRCNDSSD) [14] and a B cell epitope from HA of A/H1N1/New Caledonia/20/99 virus (aa 331-355; VTGLRNIPSIQSRGLFGAIAGFIEG; $HA_{331\text{-}355}$) were synthesized by EZbiolab (Carmel, CA). Hybridomas producing mAbs α-DEC-205, isotype control III-10, anti-CD4 and anti-M1 of influenza were cultured, and the mAb present in the supernatants was purified as previously described by *Badillo-Godinez et al.* [22]. A polyclonal anti-influenza virus Ab was produced in rabbit by immunizing with a mixture of the influenza viruses A/H1N1/New Caledonia/20/99 and A/H3N2/NT/60/68 (anti-FLU). A homemade mouse anti-OVA polyclonal Ab was described previously [22]. Anti-influenza M2e mAb 14C2 (IgG1) was purchased from ABCAM. The polyclonal secondary antibodies rabbit anti-rat IgG (Sigma), horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG1 and IgG2a-HRP (Zymed), goat anti-mouse IgG-HRP (Jackson Immuno Research) and goat anti-rabbit IgG-HRP (Dako) were used for different experimental procedures. For flow cytometry, the mAb anti-mouse CD11c (HL3)-biotin, streptavidin conjugated to APC or FITC and polyclonal goat anti-mouse IgG-FITC were

acquired from Biolegend; polyclonal goat anti-rat IgG-Alexa 488 antibody was from Life Technologies.

2.5. Virus titration

Focus-forming units (ffu) were evaluated in lung homogenates infected with IAV. Homogenates were serially diluted in DMEM medium containing 1 μg/mL of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)- treated trypsin (Sigma), and 100 μL of each dilution was used to infect MDCK-cell monolayers in 96-well plates for 1 h at 37 °C. After this time, infection medium was replaced with fresh DMEM and incubated for 16 h at 37 °C. Cells were fixed with 80% acetone in PBS and incubated with a rabbit anti-FLU polyclonal Ab for 1 h at 37 °C. Cells were washed and a goat anti-rabbit IgG-HRP polyclonal Ab was added as secondary Ab and incubated for 1 h at 37 °C. After washing, the substrate (3-amino-9-ethyl carbazole in 0.1 M acetate buffer; Sigma) was added and after 5 min at room temperature, plates were washed with distilled water. Infection foci were counted on light microscopy and expressed as ffu/mL. The lethal dose 50 (LD_{50}) of lung homogenates in mice was performed following the Reed Muench's method described elsewhere [28].

Plaque forming units (pfu) were evaluated in allantoic fluid infected with IAV. Allantoic fluid was 10-fold-diluted in DMEM medium containing 1 μg/mL of TPCK-treated trypsin, and 1 mL of each dilution was used to infect MDCK-cell monolayers in 6-well plates for 2 h at 37 °C. After this time, infection medium was discarded and 3 mL of 0.5% agar noble (Difco) in DMEM medium supplemented with penicillin/streptomycin and 1 μg/mL of TPCK-treated trypsin was overlaid to the cells. After 2–3 days of incubation at 37 °C, 1.5 mL of paraformaldehyde 2% in PBS was added to each well for 2 h at room temperature. After washing with PBS, fixed cells were stained with 1% crystal violet in ethanol for 5 min, washed with distilled water and infection plaques were counted under an inverted microscope. The infection plaques were expressed as pfu/mL.

2.6. Chemically cross-linking of monoclonal antibodies with M2e or OVA

Monoclonal antibodies were cross-linked with antigen as previously described [22]. Briefly, the mAbs α-DEC-205 and its isotype control were individually activated with succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (Pierce); while M2e peptide and ovalbumin (Sigma) were independently activated with Traut's reagent (2-iminothiolane) (Pierce). After this process, mAbs and antigens were dialyzed separately (except for the short M2e peptide) and mixed (1:1) for two days at 4 °C. After this time, the non-bound antigen was removed passing cross-linked mAbs for a 100 kDa-ultra-centrifuge filter (Amicon). The resulting conjugates, α-DEC-205:M2e, isotype:M2e and α-DEC-205:OVA were subjected to SDS-PAGE, Western blot and dot blot analysis.

2.7. Western blot

To confirm that antibodies were cross-linked with antigens, and in order to include free M2e peptide (2.7 kDa) as control for the anti-M2e mAb, they were resolved in a 10/15% SDS-PAGE To this end, a third of the total volume was loaded with 15% acrylamide and on top the rest of the volume with 10% acrylamide. Samples were resolved under non-reducing or reducing conditions, using 144 mM 2-Mercaptoethanol (2-ME), and transferred to nitrocellulose membranes (Perking Elmer) for 1 h at 200 V, using a Mini-Trans blot (Bio-Rad). Membranes were blocked with blocking buffer (5% fat-free milk and 0.05% Tween-20 in PBS) for 1 h at room temperature. After washing with 0.05% Tween-20 in PBS, the membranes were incubated for 1 h at 37 °C with a polyclonal Ab rabbit anti-rat IgG or with a mouse anti-M2e mAb (14C2) diluted 1:1000 in blocking buffer. The membranes were washed and a secondary polyclonal Ab goat anti-rabbit IgG-HRP or goat anti-mouse IgG1-HRP diluted in blocking buffer 1:2500 were added and incubated for 1 h at 37 °C. After washing, Luminol (Perking Elmer) was added to each membrane to develop signal and captured with an Odyssey Fc (LI-COR).

2.8. Slot blot

To calculate the amount of peptide M2e bound to antibodies (α-DEC-205:M2e and isotype:M2e) a slot blot assay was performed. Serial dilutions of M2e and the $HA_{331-355}$ peptide, as well as conjugates, were immobilized in a nitrocellulose membrane using the Hybri-Slot Manifold (Biometra). The membranes were treated with the Western blot procedure previously described, using the mAb mouse anti-M2e (14C2) as primary antibody and the polyclonal Ab goat anti-mouse IgG1-HRP as secondary Ab. The amount of M2e peptide bound to the conjugates was extrapolated from the standard curve of free M2e peptide. The $HA_{331-355}$ peptide was used as negative control.

2.9. Immunization and challenge

Groups of 4–6 mice were inoculated s.c. three times, 3 weeks apart with one of the following treatments: α-DEC-205:M2e, isotype:M2e or α-DEC205:OVA with the equivalent of 1 μg of antigen, 5 μg of free peptide M2e, or PBS alone, all in presence of 50 μg of poly I:C (Amershan BioSciences) as adjuvant. Before each immunization (days 0, 21 and 42), mice were bled to analyze levels of anti-M2e antibodies in sera. Two weeks after the third immunization (day 57), mice were anesthetized with isoflurane (Soflorane Vet, PiSA Laboratory) and intranasally (i.n.) challenged with 50 μL of PBS containing $30LD_{50}$ of IAV H3N2/NT/60/68 or H1N1/New Caledonia/20/99. Morbidity (weight loss) and mortality were monitored daily for 10 days. For ethical reasons, mice that lost more than 25% of their initial weight were euthanized and considered an influenza-related death.

2.10. Elimination of $CD4^+$ T cells

For evaluating the role of effector $CD4^+$ T cells on protection against IAV lethal challenge, immunized mice with α-DEC-205:M2e were inoculated intraperitoneally (i.p.) with 2.5 mg of purified anti-CD4 mAb GK1.5 on days −3, 0 and + 3 with respect to the challenge day with IAV. This treatment induced above 90% depletion of $CD4^+$ T cells in the spleen as determined by flow cytometry (data not shown). Mortality of mice was scored as indicated above.

2.11. Passive transfer of serum

Naïve mice were transferred with 350 μL of pooled sera from mice immunized with α-DEC-205:M2e (post third immunization), or pooled sera from control mice inoculated with PBS plus adjuvant (mock sera). Mortality of mice was scored as indicated above.

2.12. Detection of M2e-specific serum antibodies by ELISA

High binding 96-well plates (Costar) were coated with 400 ng per well of the M2e synthetic peptide, diluted in 0.1 M carbonate buffer (pH 9.6), overnight at 4 °C. Then, wells were blocked with blocking buffer (5% w/v fat-free milk in PBS/0.05% Tween-20) for 2 h at room temperature. After this, a pool of sera from immunized mice was diluted 1:200 for screening Ab levels regarding each immunization, or 1:500 to 1:128,000 for titration purposes, in

blocking buffer and 50 μL of the dilutions added per well and incubated for 2 h at 37 °C. Plates were washed with PBS/Tween-20 0.05% and 50 μL of secondary Ab goat anti-mouse IgG-HRP (1:2500), rabbit anti-mouse IgG1-HRP (1:500) or rabbit anti-mouse IgG2a (1:500) diluted in blocking buffer was added and incubated for 1 h at 37 °C. After washing, 50 μL of substrate (*o*-phenylenediamine; Sigma) was added and incubated for 15 min at room temperature. The reaction was stopped with H_2SO_4 2 M and plates were read at 492 nm in a 96-well plate spectrophotometer (BIOTEK, Instruments).

2.13. Influenza virus neutralization assay

Levels of neutralizing antibodies against IAV in experimental sera were analyzed by an ELISA-based antigen reduction neutralization assay (NELISA). A pool of sera from immunized mice or rabbit anti-FLU polyclonal antibody were 2-fold serially diluted with DMEM in 96-well plates, and 50 μL of each dilution was mixed with 50 μL of DMEM containing 100 pfu of IAV H1N1/New Caledonia/20/99 (allantoic fluid). After 1 h of incubation at 37 °C, the mixture was added to a monolayer of MDCK cell in tissue culture flat-bottomed 96-well plates, and incubated for 1 h at 37 °C. After this time, the inoculum was replaced with fresh DMEM with 1 μg/mL TPCK-treated trypsin and incubated for 18–20 h 37 °C. Plates went through three cycles of freezing and thawing and the viral load in the cell lysates determined by a sandwich ELISA assay. For this end, high binding 96-well plates were coated with rabbit anti-FLU polyclonal Ab (1:8000) in PBS overnight at 4 °C. Wells were blocked with blocking buffer for 2 h at room temperature and 50 μL of the cell lysates added and incubated for 1 h at 37 °C. Plates were washed and 50 μL of mouse mAb anti-IAV M1 diluted in blocking buffer (1:100) was added and incubated for 1 h at 37 °C. Finally, 50 μL of rabbit anti-mouse IgG1-HRP (1:500) diluted in blocking buffer was added and incubated for 1 h at 37 °C. Substrate addition and the plate reading was done as indicated above for the anti-M2e peptide ELISA.

2.14. Flow cytometry

Bone marrow-derived DCs were collected and washed twice with binding buffer (BB; 0.01% sodium azide, 2 mM EDTA, and 2% FBS) containing 2% of rabbit serum (for blocking Fc receptors), resuspended in the same buffer and incubated for 30 min at 4 °C. Cells were washed with BB, resuspended in 100 μL of BB containing the mAbs anti-CD11c-Biotin and α-DEC-205 and incubated for 20 min at 4 °C. After washing, cells were resuspended in 100 μL of BB containing streptavidin-APC, and a goat anti-rat IgG-Alexa 488 polyclonal Ab and incubated for 20 min at 4 °C. Finally, cells were washed, fixed with 100 μL of 2% paraformaldehyde at room temperature for 20 min and 10,000 cells per sample were acquired and analyzed with a FACS Aria-II flow cytometer (Beckton Dickinson). Gating analysis was done with Flow Jo Software (Tree Star, Inc). When the α-DEC-205 conjugates were used, they were followed by a mouse anti-M2e mAb or a mouse anti-OVA polyclonal Ab and a goat anti-mouse IgG-FITC polyclonal antibody, following the protocol described above.

2.15. Statistical analysis

One-way ANOVA, Dunnett's multiple comparison tests, and the Fisher's exact tests were performed using GraphPad Prism version 6.0 (GraphPad Software Inc, La Jolla CA). Differences in p values equal or minor to 0.05 were considered statistically significant.

3. Results

3.1. Characterization of mAbs cross-linked to antigen

Rat mAbs antibodies α-DEC-205 and the isotype control were purified from cultures of hybridomas NLD145 and III-10 respectively, and afterward, they were chemically cross-linked to the synthetic peptide M2e or OVA, as an irrelevant antigen. Free mAbs, free M2e peptide, and the conjugates were resolved in a 10/15% SDS-PAGE under non-reducing and reducing conditions and analyzed by Western blot using an anti-rat Ab. As expected, the unconjugated α-DEC-205 mAb gave a major band of 150 kDa, whereas α-DEC-205:M2e and isotype:M2e showed two predominant bands: one of around 150 and the other of 180 kDa (Fig. 1A, top). When an anti-M2e mAb was used, both bands were also detected (Fig. 1A, bottom). Although it is not shown here, α-DEC-205:OVA showed two bands of 150 and 195 kDa: the first one corresponding to the free antibody and the second one to the antibody conjugated to one molecule of OVA, as previously reported [22]. These results indicate that antibodies were successfully cross-linked to the antigens, but in the case of α-DEC-205 and the isotype control, the cross-linking to M2e turned out to be heterogeneous. The band of around 150 kDa appeared to be composed of the mAb cross-linked to few M2e peptides, although the presence of free mAb could not be discarded, and the band of 180 kDa was likely to represent the mAb cross-linked to approximately 12 molecules of the peptide. When samples were resolved under reducing conditions and developed with anti-Rat Ab, a complete reduction was observed with the α-DEC-205 mAb since only two bands of 25 and 55 kDa corresponding to the light and heavy chain, respectively, appeared (Fig. 1A, top).

On the other hand, the reduced conjugates generated the 25 and 55 kDa bands, as well as other bands around 130–160 kDa, disappearing the band around 180 kDa, suggesting that the conjugates were not reduced completely. When the anti-M2e mAb was used a similar pattern was observed but with a new discrete band of around 2.7 kDa corresponding to free M2e (Fig. 1A bottom). These results indicate that, as expected, the thioether bond between the mAb and the peptide is highly resistant to reducing agents and that the cross-linked peptide maybe protecting the disulfide bonds of the heavy and light chain. However, it is clear that both the light and the heavy chains can be cross-linked with at least one M2e peptide. Using a dot-blot analysis, it was possible to determine that the M2e peptide represented from 4% to 10% of the total mass of the conjugates (Fig. 1B).

To determine whether conjugates maintained their specificity to DEC-205, BM-derived DC were stained with mAbs against CD11c, α-DEC-205, α-DEC-205:OVA and α-DEC-205:M2e or isotype:M2e conjugates, and analyzed by flow cytometry. The α-DEC-205 mAb was found to stain about 27% of the DC when an anti-rat polyclonal Ab was used (Fig. 1C). A similar percentage of DC was stained with the α-DEC-205:OVA conjugate followed by an OVA-specific polyclonal Ab or with the α-DEC-205:M2e conjugate followed by an M2e-specific mAb. Isotype:M2e stained only at background levels. In this way, although both chains the α-DEC-205 mAb can be cross-linked with the M2e peptide, it does not have a significant effect on the specificity of the mAb to DEC-205.

3.2. Targeting M2e to DEC-205 induced an enhanced immune response compared to free M2e

To evaluate the immunogenicity of the conjugate, mice were inoculated three times s.c. with α-DEC-205:M2e, equivalent to 1 μg of the M2e peptide, in the presence of poly I:C as adjuvant.

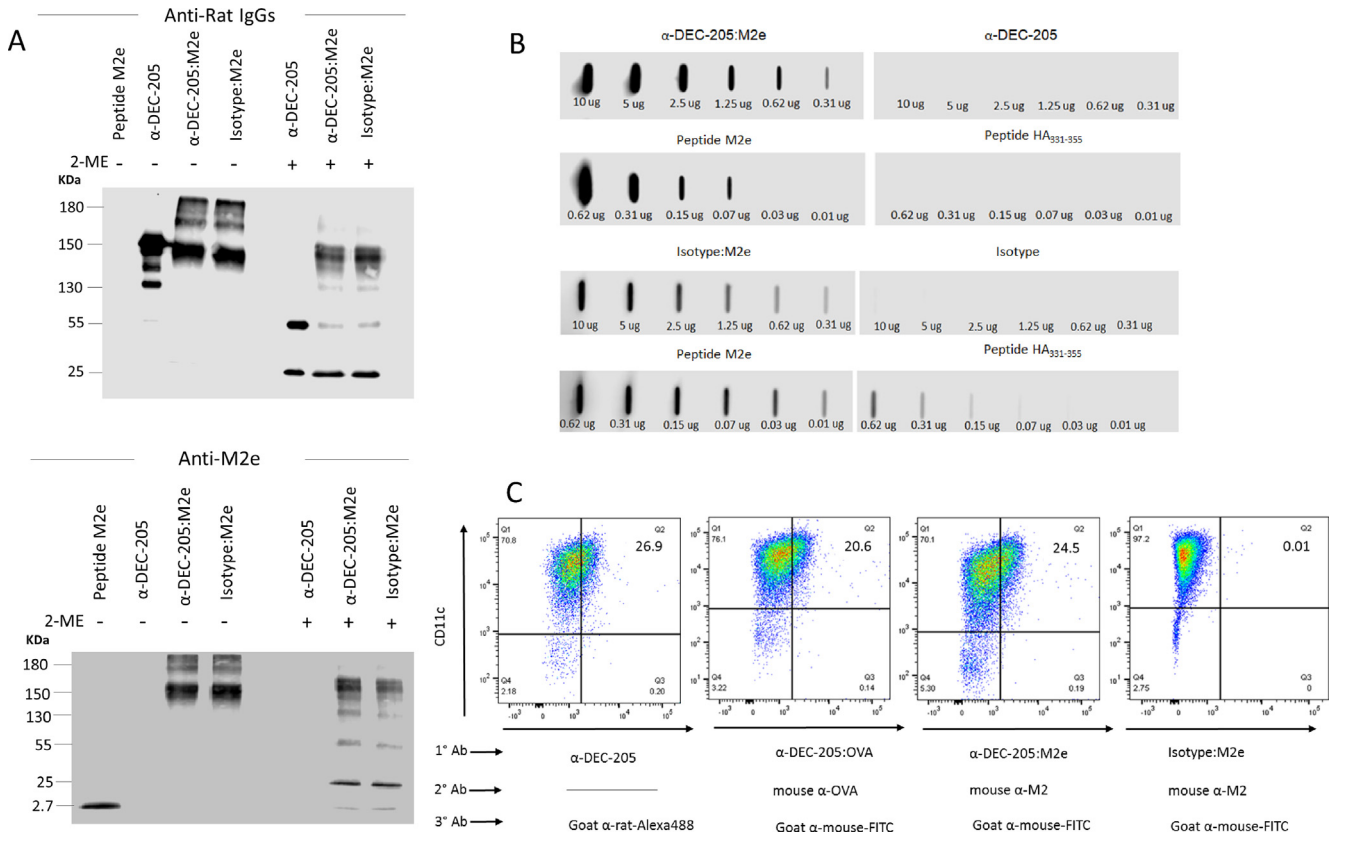

Fig. 1. Characterization of mAbs conjugated with antigen. Peptide M2e and OVA were conjugated to α-DEC-205 mAb, while isotype control was conjugated only to M2e. (A) Cross-linked mAbs were resolved in a 10/15% acrylamide gel under reducing and non-reducing conditions and analyzed by Western blot. (*top*) The membrane was treated with rabbit anti-rat IgG polyclonal Ab followed by a goat anti-rabbit-IgG polyclonal Ab-HRP; (*bottom*) The membrane was treated with a mouse anti.M2e mAb (14C2) followed by goat anti-mouse IgG1 polyclonal Ab-HRP. Signal was developed by chemiluminescence. The presence or absence of 2-ME is indicated above each lane with symbols (+ and −, respectively). (B) Serial dilutions of Abs α-DEC-205:M2e or α-DEC-205 alone *(top)* and isotype:M2e or isotype alone (*bottom*) were immobilized onto a nitrocellulose membrane that was treated with the mouse anti-M2e mAb (14C2) followed by a goat anti-mouse IgG1 polyclonal Ab-HRP. The amount of M2e peptide bound was extrapolated from the standard curve of free M2e peptide, and the $HA_{331-355}$ peptide was used as a negative control. Signal was developed by chemiluminescence and analyzed with the *ImageJ* software. (C) Bone marrow-derived DCs were used to verify the specificity of the α-DEC-205 conjugates by flow cytometry. Bone marrow-derived DCs were double stained with a mAb anti-CD11c-Biotin (followed by streptavidin-APC) an 5 μg of α-DEC-205 or its conjugates. α-DEC-205 was detected with an anti-rat IgG polyclonal Ab-Alexa 488. α-DEC-205:M2e and isotype:M2e were detected with a mouse anti-IgG1 mAb (14C2) followed by a goat anti-mouse IgG polyclonal Ab-FITC. α-DEC-OVA was detected with a mouse anti-OVA polyclonal Ab followed by a goat anti-mouse IgG polyclonal Ab-FITC.

As controls, mice were inoculated with 5 μg of free M2e peptide, isotype:M2e, α-DEC-205:OVA or PBS, also in the presence of adjuvant (Fig. 2A). The level of the anti-M2e IgG Abs was evaluated three weeks after each inoculation (at a fixed serum dilution of 1:200). The α-DEC-205:M2e conjugate was found to induce high levels of antibodies after the second inoculation. In contrast, free M2e peptide and the isotype:M2e conjugate induced antibodies only after the third inoculation (Supplementary Fig. 1). However, when the titer of the total anti-IgG antibody was analyzed after the third immunization, the α-DEC-205:M2e conjugate generated up to 10 times more antibody than the controls, and the IgG1 Abs were highly predominant over the IgG2a Abs (Fig. 2B). These results show that targeting the M2e peptide to DEC-205 induces a higher IgG antibody response than the free M2e peptide and, since the IgG1 subtype is predominant, it suggests that a higher Th2-type response is induced.

To determine whether the α-DEC-205:M2e conjugate could induce a heterosubtypic protection against the IAV infection, mice were immunized as described above. Two weeks after the last inoculation, mice were infected with a lethal dose of the IAV New Caledonia/H1N1 or NT/60/H3N2. Mice were found to show the same morbidity (evaluated as body weight loss) as the controls (Fig. 3A). However, the mice immunized with the α-DEC-205:M2e conjugate showed significant higher heterosubtypic protection levels against mortality than the controls (Fig. 3B and C). Mice immunized with the α-DEC-205:M2e conjugate showed up to 76% of survival after being infected with the IAV New Caledonia/H1N1 or NT/60/H3N2, compared to a 20% to 40% survival among mice immunized with the free M2e peptide or the isotype:M2e conjugate. Mice inoculated with the α-DEC-205:OVA conjugate or PBS did not show any protection. These results indicate that the α-DEC-205:M2e conjugate does not prevent morbidity, but it does potentiate heterosubtypic protection against mortality, in comparison with the free peptide or the isotype:M2e conjugate.

3.3. Anti-M2e serum antibodies protect against the infection unlike effector $CD4^+$ T cells

Previous studies have shown that the protective immune response induced by the M2e peptide is mainly mediated by serum Abs [29–31], although effector $CD4^+$ T cells have also been implicated [11]. In order to determine whether the serum Abs are responsible for the protection observed, mice were immunized with the α-DEC-205:M2e conjugate, and two weeks after the third inoculation, the sera were collected and transferred to naïve mice, followed by a lethal challenge of the IAV NT/60/H3N2. As positive controls, mice immunized with the conjugate were used. As negative controls, naive mice were transferred with serum from mice

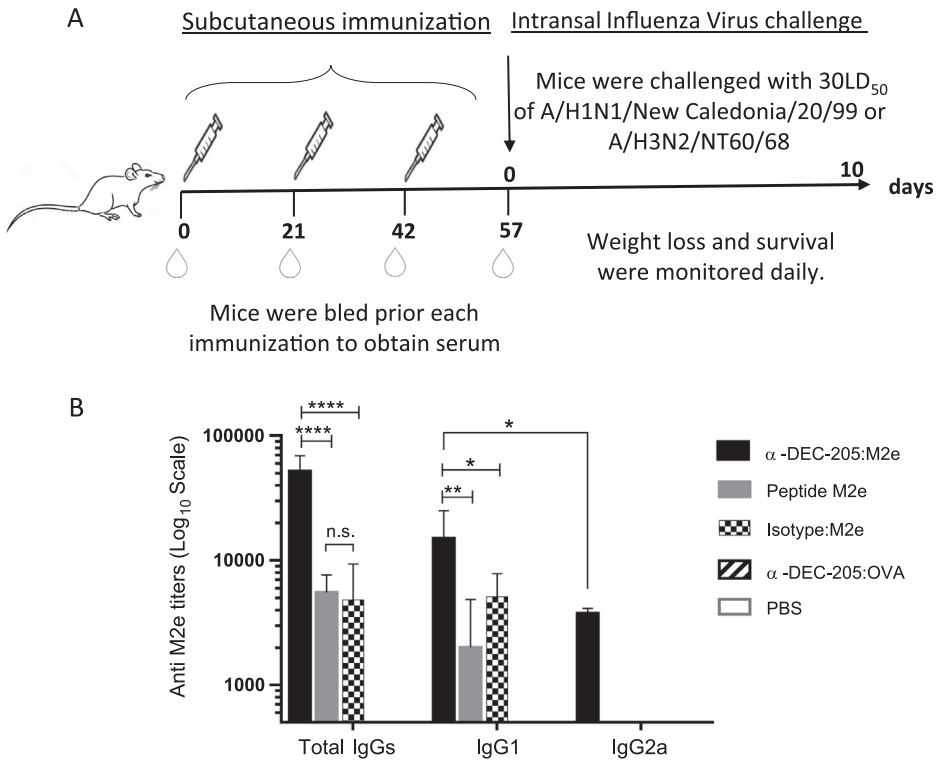

Fig. 2. Experimental design and anti-M2e Abs titers. (A) Experimental design for the scheme of vaccination, blood sampling, and IAV challenge. (B) Anti-M2e serum Abs titers. Post-third immunization sera (day 57) from vaccinated mice of every experimental group were pooled and serially diluted in PBS (1:500 to 1:128,000) and tested by ELISA for detecting specific M2e serum antibodies, as described in material and methods. Ab titers were expressed as the half-maximal value of absorbance of each experimental curve and represented as the mean ± SD of two independent experiments (each with 6 mice per group). Statistically significant differences are symbolized as *p* values (*, < 0.05; **, < 0.01; ***, < 0.001; ****, < 0.0001).

inoculated only with poli I:C or left untreated. Only the naïve mice transferred with serum from immunized mice with α-DEC-205:M2e were found to present a similar survival level as mice immunized with the same conjugate (Fig. 4). As expected, the serum antibodies from mice immunized with the α-DEC-205:M2e conjugate were non-neutralizing in an *in vitro* assay (Supplementary Fig. 2). To determine whether the $CD4^+$ T cells were also involved in the protection as effector cells, mice immunized with the α-DEC-205:M2e conjugate were depleted of this cell population with the mAb GK 1.5 and challenged with a lethal dose of the IAV NT/60/H3N2. As positive controls, mice without mAb treatment were immunized with the conjugate, and as negative controls, naïve mice were used. $CD4^+$ T cells were found not to have a role in the protection as effector cells since the immunized mice treated with the mAb showed similar levels of protection as the immunized mice without treatment (Fig. 5). These results indicate that the protection induced by the α-DEC-205:M2e conjugate is dependent on non-neutralizing serum Abs and independent of effector $CD4^+$ T cells.

4. Discussion

Seasonal IAV is responsible for half a million deaths annually, and there is a continuous concern that totally new strains emerge due to their mechanisms of evolution, causing worldwide pandemics. Despite the existence of seasonal vaccines, their efficacy depends on the accuracy of the selection of the IAV strains every year, and they may have little or no impact on pandemic viruses [1]. Consequently, interest in developing a heterosubtypic "universal vaccine" that could protect against any IAV strains, using highly conserved antigens along with strategies to increase their immunogenicity, has arisen.

In this study, the poorly immunogenic but highly conserved 24 aa ectodomain of M2 (M2e) from the IAV was targeted to DEC-205 (present mainly in DC), in the presence of poli I:C as adjuvant, in order to enhance its immunogenicity in a mouse model. To this end, M2e was chemically cross-linked to an α-DEC-205 specific mAb or to isotype control. This approach did not prevent morbidity, but it did induce up to 76% survival against a heterosubtypic lethal challenge when minimal quantities of M2e were targeted to DEC-205 (3 μg/mouse delivered in three doses). This contrasts with the 20% to 40% protection achieved in mice receiving the equivalent amount of M2e conjugated to isotype control or five times as much free M2e peptide. It is worth noting that the cross-linking of the M2e peptide to both chains of the α-DEC-205 mAb did not alter the capacity of the mAb to recognize DEC-205 on the surface of DC. The protection was dependent on non-neutralizing serum antibodies and independent of effector $CD4^+$ T cells.

DEC-205 is highly expressed on the surface of DC, and its function is to internalize antigens and deliver them to endosomes containing major histocompatibility complex (MHC) class II molecules, promoting their presentation to $CD4^+$ T cells or cross-presentation to $CD8^+$ T cells in the context of the MHC class I molecules. Therefore, targeting antigens to DEC-205 has been used mostly to induce T cell responses, either inflammatory for infectious diseases (with adjuvant) or tolerogenic for autoimmune diseases (without adjuvant) [21,22,25,32–38]. However, several studies have shown that targeting antigens to DEC-205 can induce an enhanced protective antibody response [39–42].

Ab levels against M2 are low or absent in response to the IAV infection in humans [14], and their role in protecting against a natural infection has yet not been elucidated. However, based on experimental studies of several M2e-based vaccination approaches

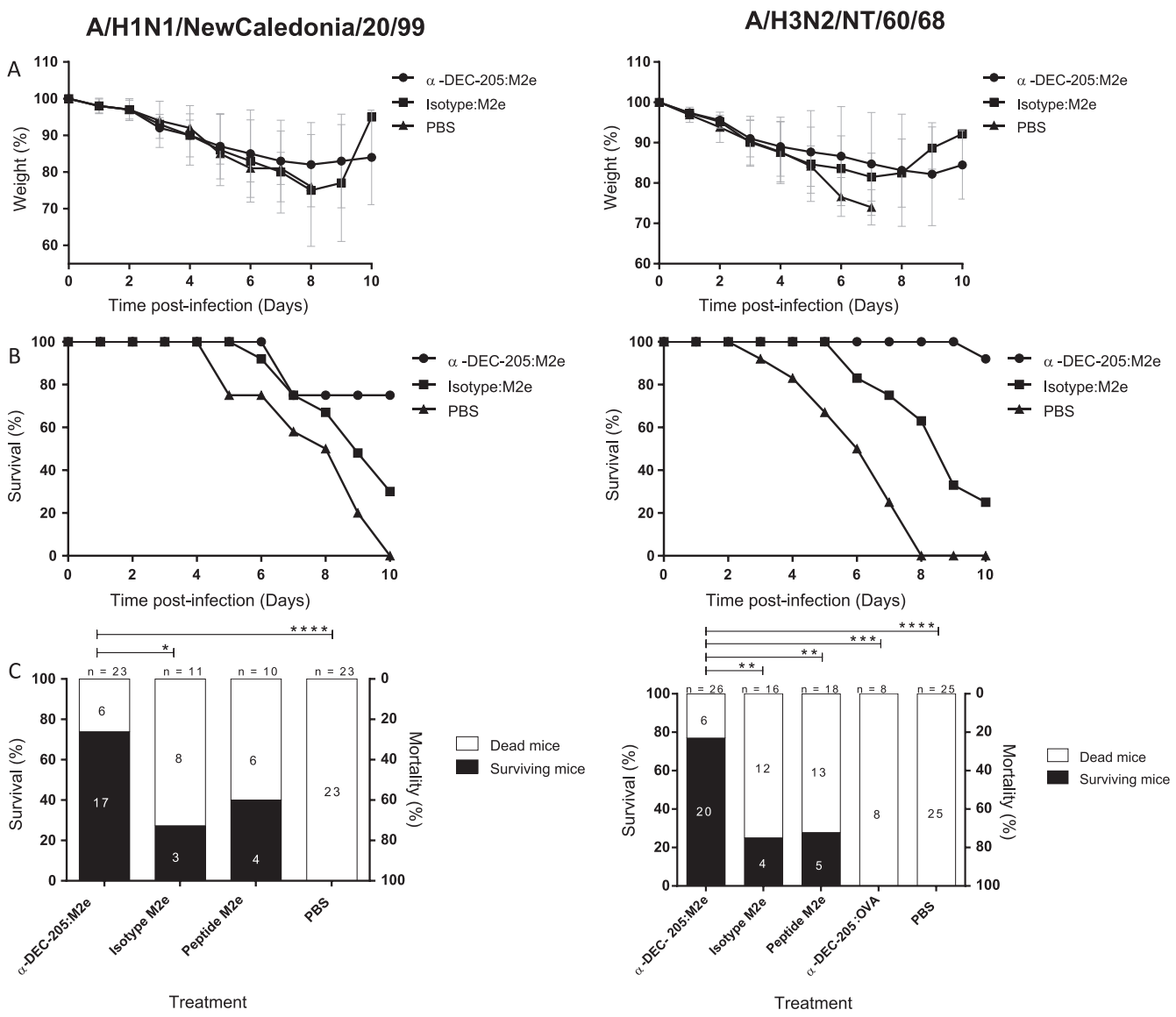

Fig. 3. Targeting M2e to DEC-205 enhances heterosubtypic protection against Influenza A Viruses. Immunized mice were challenged i.n. with 30LD_{50} of A/H3N2/NT/60/68 or A/H1N1/New Caledonia/20/99 on day 57 according to the scheme of vaccination. Weight loss (A) and survival (B) percentage curves were constructed with data of two independent experiments (each with 6 mice per group). Vertical bars indicate the standard deviation of the mean of weight each day for every experimental group. (C) Overall survival of 2 to 5 experiments against IAV H1N1/New Caledonia/20/99 (*left*) and H3N2/NT/60/68 (*right*), represented as cumulative absolute numbers of surviving and non-surviving mice, graphed in stacked bars normalized to 100%. Statistically significant differences are symbolized as *p* values (*, < 0.05; **, < 0.01; ***, < 0.001; ****, < 0.0001).

in animal models, it has been determined that specific M2e-Abs are extremely cross-reactive, and they induce high levels of protection against multiple influenza strains [30]. Although M2e-Abs are non-neutralizing, they contribute to the protection by binding to the M2 exposed on the surface of infected cells and by cooperating with macrophages and NK cells via their Fcϒ receptors (FcRϒ) [7,8,43,44]. Accordingly, targeting M2e to DEC-205 in our work significantly increased the production of specific anti-M2e IgG serum antibodies, and, as expected, they were non-neutralizing *in vitro*. Nonetheless, M2e-Abs turned out to be the critical protective effector mechanism of this vaccine, as evidenced by serum transference experiments.

Ab subtypes IgG1 and IgG2a have been associated with the protection induced by M2e since both subtypes are able to bind FcRϒ [7]. IgG2a can bind FcRϒI, III, and IAV, whereas IgG1 only binds FcRϒIII [43]. Previous studies on the immunogenicity of M2e using different vaccine platforms have shown variable results with respect to the predominance of IgG1- and IgG2a-M2e specific Abs [10,45–50]. In our model, anti-M2e IgG1 Abs were predominant over IgG2a antibodies in mice inoculated with the α-DEC-205:M2e conjugate, isotype control or with free M2e peptide. Therefore, it is likely that the protective activity of the serum from mice inoculated with the α-DEC-205:M2e conjugate was due mainly to the action of the anti-M2e IgG1 Abs. Thus, it would be expected that the 20–40% protection observed in mice inoculated with isotype.M2e or with free M2e peptide was also due the anti-M2e IgG1 Abs, as they presented very low levels of anti-M2e IgG2a Abs. In fact, in preliminary results (data not shown), we found that sera from these mice can also induce protection against IVA infection in naïve mice.

Contrary to our results, previous reports have indicated that, under certain conditions, the protection induced by M2e depends mainly on pro-inflammatory effector $CD4^+$ T cells [11]. The ratio IgG1: IgG2a found in our study suggests that a Th2-type response was predominant compared to a pro-inflammatory Th1-type response. This could explain the fact that the protection against

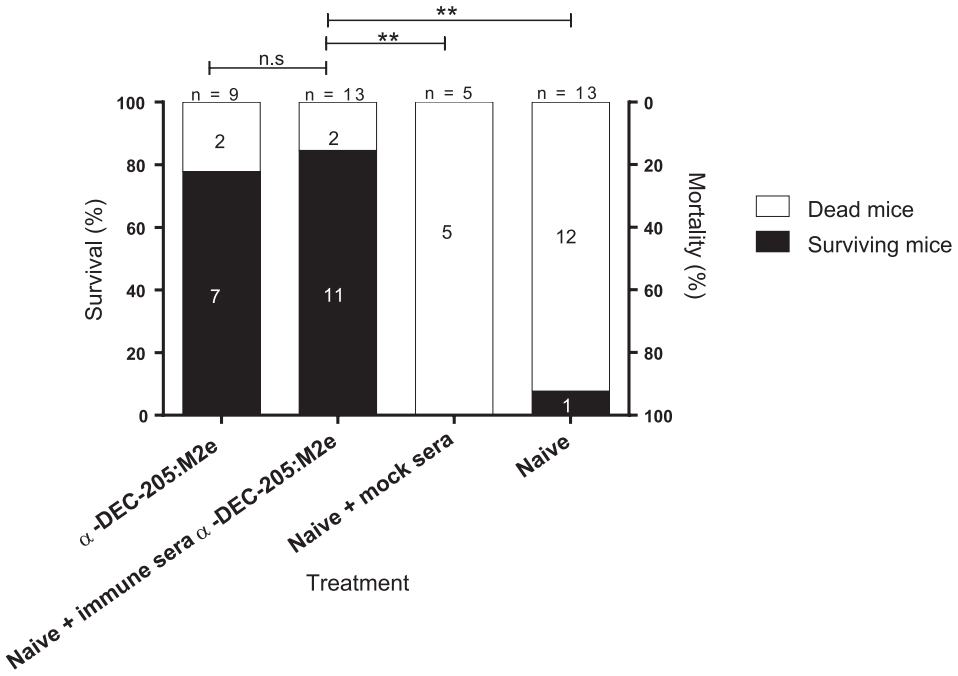

Fig. 4. Adoptive transference of serum anti-M2e Abs induces protection against IAV infection. Overall survival against lethal challenge with $30LD_{50}$ of A/H3N2/NT/60/68. Experimental groups included: naïve mice that were transferred i.p. with 350 μL/mouse of a pooled sera obtained from mice immunized with α-DEC-205:M2e (post third immunization) (the titers of anti-M2e Abs were; IgG: 1:52,000, IgG1: 1:15,000 and IgG2a: 1:3,750); as negative controls: naïve mice receiving mock sera (immunized with PBS plus poly I:C) and naive mice without transference of sera; as a positive control of protection, mice immunized with α-DEC-205:M2e were used. The figure represents the overall survival of 1 to 3 independent experiments represented as cumulative absolute numbers of surviving and non-surviving mice, graphed in stacked bars normalized to 100%. Statistically significant differences are symbolized as *p* values (*, < 0.05; **, < 0.01; ***, < 0.001; ****, < 0.0001).

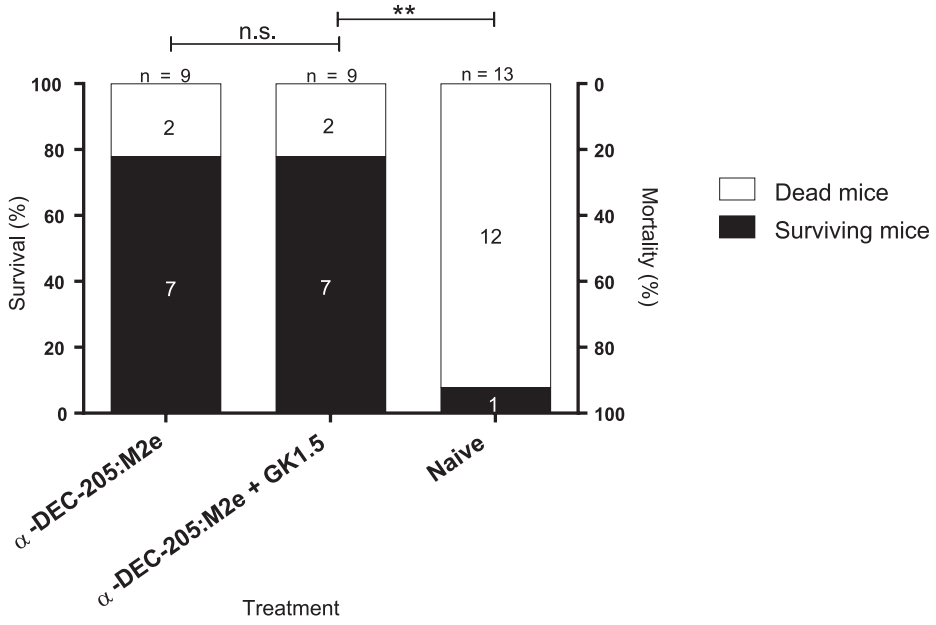

Fig. 5. Effector $CD4^+$ T cells are not necessary for protection when M2e is targeted to DEC-205. Overall survival against lethal challenge with $30LD_{50}$ of A/H3N2/NT/60/68. Experimental groups included: mice immunized with α-DEC-205:M2e; mice immunized with α-DEC-205:M2e and injected i.p. with three doses of 2.5 mg of purified anti-CD4 (GK1.5) mAb on days −3, 0 and + 3 with respect to challenging day, and the naïve group without treatment. The figure represents the overall survival of 2 to 3 independent experiments represented as cumulative absolute numbers of surviving and non-surviving mice, graphed in stacked bars normalized to 100%. Statistically significant differences are symbolized as *p* values (*, < 0.05; **, < 0.01; ***, < 0.001; ****, < 0.0001).

the IAV infection observed in mice inoculated with the α-DEC-205:M2e conjugate was not abrogated by the *in vivo* depletion of $CD4^+$ T cells previous to the IAV challenge. This suggests a major role of $CD4^+$ T cells as helper cells for antibody production.

In our model, high titers of serum anti-M2e Abs were present after the second immunization with the α-DEC-205:M2e conjugate, whereas only low titers of serum Abs were detected after the third inoculation in the group of mice immunized with

the isotype:M2e conjugate. Previous studies have shown that the M2e peptide contains both B and T cell epitopes within its short sequence [51]. More specifically, M2e contains a $CD4^+$ T cell epitope [11,13] restricted to the MHC class II molecule IA^d, which is present in the BALB/c ($H\text{-}2^d$) mice used in this work. Thus, it is likely that the enhanced Ab response in mice immunized with the α-DEC-205:M2e conjugate was the result of a very efficient induction of anti-M2e $CD4^+$ T cells by DC via DEC-205 [25], which subsequently cooperated with M2e-specific B cells. Thus, the α-DEC-205:M2e conjugate would not only play a role targeting M2e to DC but would also act as a carrier complex so specific B cell receptors are able to recognize M2e. After processing, these cells would present the M2e-derived peptides to memory $CD4^+$ T cells in the context of the MHC class II molecules.

In contrast, although the isotype:M2e conjugate could also act as a carrier complex to stimulated B cells, the low Abs titers observed could be explained by a poor induction of memory $CD4^+$ T cells. Accordingly, Amorin et al. showed the importance of having B and T cells epitopes within a peptide sequence targeted to DEC-205 in order to induce strong specific antibody response [41], since when they targeted the polypeptide MSP_{42} from Plasmodium vivax to DEC-205, which contains both B and T cell epitopes, a strong antibody response was induced. On the other hand, a fragment of this polypeptide containing only B cell epitopes (MSP_{19}) failed to do so.

The role of DEC-205 on activated B cells during the induction of high titers of serum Abs in mice immunized with the α-DEC-205:M2e conjugate should not be discarded. Although B cells poorly express DEC-205 compared to DC, its expression is enhanced when they become activated, as is their potential to capture and present peptides to T cells [52]. Moreover, germinal center B cells targeted with antigen via DEC-205 increase their potential to interact with Tfh and, therefore, to enhance the generation of plasma cells [23,24]. Thus, it is likely that the α-DEC-205:M2e conjugate not only stimulated B cells through antigen-specific receptors but also targeted M2e to DEC-205, present on the activated B cells, which resulted in an increased antigen presentation to memory CD4 T cells and the generation of plasma cells.

Finally, targeting M2e to DEC-205 seems to be an efficient way to enhance the protective immune response against heterosubtypic infections by IAV, which provides a new alternative for the development of heterosubtypic "universal vaccines." Future experiments are needed to identify the precise immunological mechanisms involved.

Acknowledgments

We thank Gerardo Arreguin and Nancy Arias (Facultad de Medicina, UAEM), and Elizabeth Mata and Graciela Cabeza (Instituto de Biotecnología, UNAM) for the excellent animal house conditions. Carlos Arias for his neutral and critical point of view of the work. Erika Melchy and Carlos Mojica for their technical assistance in the flow cytometry assays. Juan Carlos Villegas-Garcia for technical assistance. This work was supported by grants from SEP-CONACYT (2015-01-257420) and SEP-PRODEP (Redes tematicas de colaboracion de CA, 2015). B.G.O is a Postdoctoral fellow of the Graduate Science Program, INSP, and was supported by CONACYT. P.Q.H.O is a Ph.D. student of the Biochemical Sciences Program, Instituto de Biotecnología, UNAM, and was supported by a CONACYT Scholarship (375463).

Contribution

E.G.F., P.Q.H.O. and L.G.D.V. conceived the study. P.Q.H.O. carried out the immunization and IAV challenge experiments, participated in the rest of the experimental techniques employed, and drafted the manuscript. B.G.O. and G.X.L. participated in the characterization of mAb-Ag conjugates. L.A.J.D. and A.B.Y. assisted in the derivation of BM-DC and other *in vitro* assays. M.H.J.L. participated in the culture and purification of IAV and other techniques. L.G.D.V. trained the first author in most of the techniques employed and advised the experimental work. E.G.F. directed the project and supervised the manuscript preparation. All authors participated in data interpretation and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2019.02.050.

References

[1] Iuliano AD et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. Lancet 2018;391(10127):1285–300.
[2] Neirynck S et al. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 1999;5(10):1157–63.
[3] Fiers W et al. M2e-based universal influenza A vaccine. Vaccine 2009;27(45):6280–3.
[4] Cho KJ et al. Structure of the extracellular domain of matrix protein 2 of influenza A virus in complex with a protective monoclonal antibody. J Virol 2015;89(7):3700–11.
[5] Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. Cell 1992;69(3):517–28.
[6] Henkel JR et al. Influenza M2 proton channel activity selectively inhibits trans-Golgi network release of apical membrane and secreted proteins in polarized Madin-Darby canine kidney cells. J Cell Biol 2000;148(3):495–504.
[7] Lee YN et al. Fc receptor is not required for inducing antibodies but plays a critical role in conferring protection after influenza M2 vaccination. Immunology 2014;143(2):300–9.
[8] El Bakkouri K et al. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J Immunol 2011;186(2):1022–31.
[9] Simhadri VR et al. A human anti-M2 antibody mediates antibody-dependent cell-mediated cytotoxicity (ADCC) and cytokine secretion by resting and cytokine-preactivated natural killer (NK) cells. PLoS One 2015;10(4):e0124677.
[10] Lee YT et al. Intranasal vaccination with M2e5x virus-like particles induces humoral and cellular immune responses conferring cross-protection against heterosubtypic influenza viruses. PLoS One 2018;13(1):e0190868.
[11] Eliasson DG et al. M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection. Mucosal Immunol 2018;11(1):273–89.
[12] Mozdzanowska K et al. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. Vaccine 2003;21(19–20):2616–26.
[13] Eliasson DG et al. CTA1-M2e-DD: a novel mucosal adjuvant targeted influenza vaccine. Vaccine 2008;26(9):1243–52.
[14] Feng J et al. Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. Virol J 2006;3:102.
[15] Gao X et al. Enhanced Influenza VLP vaccines comprising matrix-2 ectodomain and nucleoprotein epitopes protects mice from lethal challenge. Antiviral Res 2013;98(1):4–11.
[16] Tompkins SM et al. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg Infect Dis 2007;13(3):426–35.
[17] Macri C et al. Targeting dendritic cells: a promising strategy to improve vaccine effectiveness. Clin Transl Immunol. 2016;5(3):e66.
[18] Inaba K et al. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. I. Expression on dendritic cells and other subsets of mouse leukocytes. Cell Immunol 1995;163(1):148–56.
[19] Witmer-Pack MD et al. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. II. Expression in situ in lymphoid and nonlymphoid tissues. Cell Immunol 1995;163(1):157–62.
[20] Shrimpton RE et al. CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. Mol Immunol 2009;46(6):1229–39.
[21] Bonifaz L et al. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major

histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med 2002;196(12):1627–38.
[22] Badillo-Godinez O et al. Targeting of rotavirus VP6 to DEC-205 induces protection against the infection in mice. Vaccine 2015;33(35):4228–37.
[23] Victora GD et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 2010;143(4):592–605.
[24] Pasqual G, Angelini A, Victora GD. Triggering positive selection of germinal center B cells by antigen targeting to DEC-205. Methods Mol Biol 2015;1291:125–34.
[25] Bonifaz LC et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med 2004;199(6):815–24.
[26] Brown EG. Increased virulence of a mouse-adapted variant of influenza A/FM/1/47 virus is controlled by mutations in genome segments 4, 5, 7, and 8. J Virol 1990;64(9):4523–33.
[27] Narasaraju T et al. Adaptation of human influenza H3N2 virus in a mouse pneumonitis model: insights into viral virulence, tissue tropism and host pathogenesis. Microbes Infect 2009;11(1):2–11.
[28] Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World J Virol 2016;5(2):85–6.
[29] Treanor JJ et al. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J Virol 1990;64(3):1375–7.
[30] Kim YJ et al. Roles of antibodies to influenza A virus hemagglutinin, neuraminidase, and M2e in conferring cross protection. Biochem Biophys Res Commun 2017;493(1):393–8.
[31] Schepens B, De Vlieger D, Saelens X. Vaccine options for influenza: thinking small. Curr Opin Immunol 2018;53:22–9.
[32] Bozzacco L et al. HIV gag protein is efficiently cross-presented when targeted with an antibody towards the DEC-205 receptor in Flt3 ligand-mobilized murine DC. Eur J Immunol 2010;40(1):36–46.
[33] Do Y et al. Induction of pulmonary mucosal immune responses with a protein vaccine targeted to the DEC-205/CD205 receptor. Vaccine 2012;30(45):6359–67.
[34] Gurer C et al. Targeting the nuclear antigen 1 of Epstein-Barr virus to the human endocytic receptor DEC-205 stimulates protective T-cell responses. Blood 2008;112(4):1231–9.
[35] Leung CS et al. Robust T-cell stimulation by Epstein-Barr virus-transformed B cells after antigen targeting to DEC-205. Blood 2013;121(9):1584–94.
[36] Maaske A et al. Mucosal expression of DEC-205 targeted allergen alleviates an asthmatic phenotype in mice. J Control Release 2016;237:14–22.
[37] Petzold C et al. Targeted antigen delivery to DEC-205(+) dendritic cells for tolerogenic vaccination. Rev Diabet Stud 2012;9(4):305–18.
[38] Wadwa M et al. Targeting antigens to Dec-205 on dendritic cells induces immune protection in experimental colitis in mice. Eur J Microbiol Immunol (Bp) 2016;6(1):1–8.
[39] Coconi-Linares N et al. Targeting of envelope domain III protein of DENV type 2 to DEC-205 receptor elicits neutralizing antibodies in mice. Vaccine 2013;31(19):2366–71.
[40] Jauregui-Zuniga D et al. Targeting antigens to Dec-205 on dendritic cells induces a higher immune response in chickens: Hemagglutinin of avian influenza virus example. Res Vet Sci 2017;111:55–62.
[41] Amorim KN et al. The presence of T cell epitopes is important for induction of antibody responses against antigens directed to DEC205(+) dendritic cells. Sci Rep 2016;6:39250.
[42] Boscardin SB et al. Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. J Exp Med 2006;203(3):599–606.
[43] Van den Hoecke S et al. Hierarchical and Redundant Roles of Activating FcgammaRs in Protection against Influenza Disease by M2e-Specific IgG1 and IgG2a Antibodies. J Virol 2017:91(7).
[44] Jegerlehner A et al. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. J Immunol 2004;172(9):5598–605.
[45] Tao W et al. Consensus M2e peptide conjugated to gold nanoparticles confers protection against H1N1, H3N2 and H5N1 influenza A viruses. Antiviral Res 2017;141:62–72.
[46] Guo Y et al. Highly conserved M2e and hemagglutinin epitope-based recombinant proteins induce protection against influenza virus infection. Microbes Infect 2017;19(12):641–7.
[47] Schotsaert M et al. Natural and long-lasting cellular immune responses against influenza in the M2e-immune host. Mucosal Immunol 2013;6(2):276–87.
[48] Rappazzo CG et al. Recombinant M2e outer membrane vesicle vaccines protect against lethal influenza A challenge in BALB/c mice. Vaccine 2016;34(10):1252–8.
[49] Wu F et al. Characterization of immunity induced by M2e of influenza virus. Vaccine 2007;25(52):8868–73.
[50] Herve PL et al. A novel subnucleocapsid nanoplatform for mucosal vaccination against influenza virus that targets the ectodomain of matrix protein 2. J Virol 2014;88(1):325–38.
[51] Deng L et al. M2e-Based Universal Influenza A Vaccines. Vaccines (Basel) 2015;3(1):105–36.
[52] Kamphorst AO et al. Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. J Immunol 2010;185(6):3426–35.