

Integrase defective lentiviral vector as a vaccine platform for delivering Influenza antigens

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

AG, ACara and DN designed the experiments, analyzed the data and wrote the paper; AG, MB, RB, FG, LC, LP, SC, DM, VM, AC and ZM performed experiments; MFV performed statistical analysis; MRC analyzed the data and critically edited the manuscript; NT provided technical knowhow on pseudotype production and critically edited the manuscript; MS provided key reagents and critically edited the manuscript. All authors have contributed to the drafting of the manuscript, have revised the work and have approved the final version.

Keywords

Lentiviral vector, influenza, Vaccine, antibody, T cell response

Abstract

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Viral vectors represent an attractive technology for vaccine delivery. We exploited the integrase defective lentiviral vector (IDLV) as a platform for delivering relevant antigens within the context of the ADITEC collaborative research program. In particular, Influenza virus hemagglutinin (HA) and nucleoprotein (NP) were delivered by IDLVs while H1N1 A/California/7/2009 subunit vaccine (HAp) with or without adjuvant was used to compare the immune response in a murine model of immunization. In order to maximize the antibody response against HA, both IDLVs were also pseudotyped with HA (IDLV-HA/HA and IDLV-NP/HA, respectively). Groups of CB6F1 mice were immunized intramuscularly with a single dose of IDLV-NP/HA, IDLV-HA/HA, HAp alone or with HAp together with the systemic adjuvant MF59. Six months after the vaccine prime all groups were boosted with HAp alone. Cellular and antibody responses to influenza antigens were measured at different time points after the immunizations. Mice immunized with HA-pseudotyped IDLVs showed similar levels of anti-H1N1 IgG over time, evaluated by ELISA, which were comparable to those induced by HAp+MF59 vaccination, but significantly higher than those induced by HAp alone. The boost with HAp alone induced an increase of antibodies in all groups, and the responses were maintained at higher levels up to 18 weeks post-boost. The antibody response was functional and persistent overtime, capable of neutralizing virus infectivity, as evaluated by hemagglutination inhibition and microneutralization assays. Moreover, since neuraminidase (NA)-expressing plasmid was included during IDLV preparation, immunization with IDLV-NP/HA and IDLV-HA/HA also induced functional anti-NA antibodies, evaluated by Enzyme-Linked Lectine Assay (ELLA). IFN γ -ELISPOT showed evidence of HA-specific response in IDLV-HA/HA immunized animals and persistent NP-specific CD8 $^{+}$ T cell response in IDLV-NP/HA immunized mice. Taken together our results indicate that IDLV can be harnessed for producing a vaccine able to induce a comprehensive immune response, including functional antibodies directed towards HA and NA proteins present on the vector particles in addition to a functional T cell response directed to the protein transcribed from the vector.

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: Yes

Please provide the complete ethics statement for your manuscript. Note that the statement will be directly added to the manuscript file for peer-review, and should include the following information:

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Animals were maintained under specific pathogen-free conditions in the animal facilities at the Istituto Superiore di Sanità (ISS) and treated according to European Union guidelines and Italian legislation (Decreto Legislativo 26/2014). All animal studies were authorized by the Italian Ministry of Health and reviewed by the Service for Animal Welfare at ISS (Authorization n. 314/2015-PR of 30/04/2015). All animals were euthanized by CO₂ inhalation using approved chambers, and efforts were made to minimize suffering and discomfort.

In review

1 **Integrase Defective Lentiviral vector as a vaccine platform for delivering Influenza antigens**

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1 **ABSTRACT**

2
3 Viral vectors represent an attractive technology for vaccine delivery. We exploited the integrase
4 defective lentiviral vector (IDLV) as a platform for delivering relevant antigens within the context of
5 the ADITEC collaborative research program. In particular, Influenza virus hemagglutinin (HA) and
6 nucleoprotein (NP) were delivered by IDLVs while H1N1 A/California/7/2009 subunit vaccine
7 (HAp) with or without adjuvant was used to compare the immune response in a murine model of
8 immunization. In order to maximize the antibody response against HA, both IDLVs were also
9 pseudotyped with HA (IDLV-HA/HA and IDLV-NP/HA, respectively). Groups of CB6F1 mice were
10 immunized intramuscularly with a single dose of IDLV-NP/HA, IDLV-HA/HA, HAp alone or with
11 HAp together with the systemic adjuvant MF59. Six months after the vaccine prime all groups were
12 boosted with HAp alone. Cellular and antibody responses to influenza antigens were measured at
13 different time points after the immunizations. Mice immunized with HA-pseudotyped IDLVs showed
14 similar levels of anti-H1N1 IgG over time, evaluated by ELISA, which were comparable to those
15 induced by HAp+MF59 vaccination, but significantly higher than those induced by HAp alone. The
16 boost with HAp alone induced an increase of antibodies in all groups, and the responses were
17 maintained at higher levels up to 18 weeks post-boost. The antibody response was functional and
18 persistent overtime, capable of neutralizing virus infectivity, as evaluated by hemagglutination
19 inhibition and microneutralization assays. Moreover, since neuraminidase (NA)-expressing plasmid
20 was included during IDLV preparation, immunization with IDLV-NP/HA and IDLV-HA/HA also
21 induced functional anti-NA antibodies, evaluated by Enzyme-Linked Lectine Assay (ELLA). IFN γ -
22 ELISPOT showed evidence of HA-specific response in IDLV-HA/HA immunized animals and
23 persistent NP-specific CD8⁺ T cell response in IDLV-NP/HA immunized mice. Taken together our
24 results indicate that IDLV can be harnessed for producing a vaccine able to induce a comprehensive
25 immune response, including functional antibodies directed towards HA and NA proteins present on
26 the vector particles in addition to a functional T cell response directed to the protein transcribed from
27 the vector.

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30 **Keywords: lentiviral vector, influenza, vaccine, antibody, T cell response**

1 INTRODUCTION

2
3 Improvements on existing delivery systems are a significant aspect to be considered in order to obtain
4 more effective vaccines. Viral vectors represent an attractive platform for vaccine development due
5 to their ability to effectively deliver antigens of interest into cells and to generate humoral and cellular
6 mediated immune responses against the encoded transgenes. Integrase defective lentiviral vectors
7 (IDLVs) represent a promising platform for immunogen delivery for vaccination purposes (1, 2).
8 IDLVs are self-inactivating, non-integrating and non-replicating vectors with high transduction
9 efficiency both *in vitro* and *in vivo*. In contrast to parental lentiviral vector (LV), IDLV are produced
10 by incorporating a mutated form of the Integrase protein in the recombinant LV, preventing integration
11 and overcoming the risk of insertional mutagenesis. The loss of integration has been demonstrated in
12 several murine models *in vivo* and *in vitro* (3). In the absence of integration, transgene expression is
13 due to the unintegrated circular forms of the vector, which are maintained episomally in the target
14 cells in the absence of cell division (4, 5). Only the transgene of interest is expressed from episomal
15 IDLV in the absence of any other parental viral product. Dendritic cells and macrophages, the main
16 cell types mediating the immune response, are non-dividing cells that are readily transduced by IDLV,
17 eliciting the expansion of antigen-specific T cells (6, 7).

18
19 Over the course of the past decade, several reports have shown that a single immunization with IDLV-
20 vectored antigens induces a persistent immune response both in murine and simian models of
21 immunization (1, 2, 8). Antigen presentation persisted for at least 30 days from immunization (9),
22 suggesting that prolonged expression might be a unique characteristic of IDLV *in vivo*. In preclinical
23 challenge experiments, a single immunization with IDLV expressing human papillomavirus (HPV)-
24 E7 tumor specific antigen resulted in eradication of established tumors in mice, validating the ability
25 to induce an effective T cell response (10). Administration of IDLV encoding antigens in murine
26 models of West Nile Virus and malaria induced protective antibodies when challenged with the
27 respective pathogens (11, 12). IDLV expressing the influenza virus nucleoprotein (NP) was protective
28 against homologous and heterosubtypic influenza virus challenge (13). More recently we showed that
29 immunization of rhesus macaques with IDLV expressing HIV-Env 1086.C gp140 induced broad and
30 sustained immune responses up to 1 year from the immunization (8). Importantly, IDLV is under
31 evaluation in clinical trials for cancer immunotherapy (ClinicalTrials.gov Identifier numbers:
32 NCT02609984, NCT02122861, NCT02387125).

33
34 In addition to the potential for inducing a prolonged immune response due to expression of the
35 vectored transgene from episomal DNA circles, IDLV can be harnessed as a cargo for delivering
36 immunogens after incorporation into the vector's particles. This can be accomplished by fusion of
37 foreign antigens with proteins incorporated into the lentiviral particles during particle assembly (14,
38 15) or via pseudotyping. Pseudotyping with heterologous viral glycoprotein envelopes is always used
39 during LV production for allowing transduction of target cells or tissues (16). LV particles can be
40 pseudotyped with a wide range of heterologous viral envelope proteins, including Influenza virus
41 hemagglutinin (HA) (17-19). Recovered particles gain the tropism of the virus from which the
42 envelope glycoprotein was derived. The most widely used envelope glycoprotein for pseudotyping
43 LV is the vesicular stomatitis G glycoprotein (VSV.G), which allow broad and efficient transduction
44 of target cells *in vitro* and *in vivo* (20). Importantly, the envelope glycoprotein displayed on the surface
45 of the particles can elicit humoral immune responses that can be protective in animal models of
46 immunizations (21-23).

47
48 Seasonal influenza A virus (IAV) infections cause significant morbidity and mortality worldwide and
49 remain a major public health concern (24, 25). Currently licensed influenza vaccines elicit
50 neutralizing antibodies (Abs) targeting HA, preventing influenza virus entry into cells (26). In
51 particular, HAs from influenza A(H1N1)pdm09 virus circulating in humans are a major antigenic

1 component contained in the annual vaccine formulations (27). However, seasonal vaccines do not
2 protect against new mismatched strains and require frequent reformulation based on the prediction of
3 strains that may circulate (27). Conversely, cell-mediated immunity targeting conserved antigens,
4 such as influenza nucleoprotein (NP), is cross reactive and, although T cell immunity is unable to
5 prevent disease, may contribute to improved clearance and decreased symptoms (28-30). NP is 90%
6 conserved among influenza virus strains (31), and it is the major target of the cross-protective T cell
7 response against influenza virus in the mouse model (32-35). However, while protection from
8 influenza challenge in mice can be achieved in presence of NP-specific T cell responses (36), an
9 efficient influenza vaccine for humans should be able to generate a more comprehensive and durable
10 immune response in terms of both protective antibodies and effective T cells.
11 To this aim, In the present study we evaluated the immune response induced by a multi-antigen
12 IDLV-based influenza vaccine pseudotyped with HA protein from A/California/7/09 (H1N1-pdm09)
13 virus and expressing either influenza HA or NP proteins as transgenes. Results indicated that a single
14 immunization with multivalent IDLVs induced persistent and effective antiviral Abs and T cell
15 responses directed towards HA or NP transgenes.
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In review

1 MATERIALS AND METHODS

2
3 **Vector construction.** The self-inactivating (SIN) lentiviral transfer vector plasmid pTY2-NP
4 expressing the nucleoprotein (NP, [GenBank: AAM75159.1](#)) from Influenza virus A/PR/8/1934
5 (H1N1) has been already described (13). For construction of SIN lentiviral transfer vector plasmid
6 pGAE-HA expressing the hemagglutinin (HA, [GenBank: ACP44189.1](#)) from influenza virus
7 A/California/7/2009 (H1N1), the codon optimized HA open reading frame (ORF) was chemically
8 synthesized, inserted into pUC57 plasmid (TwinHelix, Milan, Italy), excised with AgeI/SalI
9 restriction enzymes and cloned into pGAE-GFP lentiviral transfer vector (376) by replacing the green
10 fluorescent protein (GFP) coding sequence. The Integrase (IN) defective packaging plasmid,
11 producing the viral proteins necessary for vector particle production, and the pHCMV-VSV.G
12 plasmid, encoding the pseudotyping vesicular stomatitis virus envelope glycoprotein G (VSV.G)
13 from Indiana serotype, necessary for IDLV entry into target cells, have been previously described
14 (387, 398). Plasmids pCAGGS-TMPRSS2 and pCAGGS-HAT, expressing the transmembrane
15 protease serine 2 (TMPRSS2, [GenBank: U75329](#)) and the human airway trypsin (HAT, [GenBank:](#)
16 [AB002134](#)) proteins respectively, were described previously (4039). Plasmids pCMV3-H1N1-C-NA
17 (Sino Biological Inc., North Wales, PA, USA), expressing the Neuraminidase (NA, [GenBank:](#)
18 [ACP41107.1](#)) derived from influenza virus A/California/4/2009 (H1N1), and pCMV-HACal09,
19 expressing the codon optimized HA protein from the CMV promoter, were used during production
20 of HA-pseudotyped IDLV-NP/HA.

21
22 **Production of lentiviral vectors.** Lenti-X human embryonic kidney 293T cell line was obtained
23 from Clontech (Mountain View, CA, USA) and was used for IDLV production following established
24 protocols (410). Cells were maintained in Dulbecco's Modified Eagles (DMEM) (Gibco, Life
25 Technologies Italia, Monza, Italy) supplemented with 10% fetal calf serum (Corning, Mediatech inc.
26 Manassas, VA, USA) and 100 units/ml penicillin/streptomycin/glutamine (PSG) (Gibco, Life
27 Technologies Italia, Monza, Italy). For production of IDLVs pseudotyped with HA and expressing
28 either HA (IDLV-HA/HA) or NP (IDLV-NP/HA), 3.5×10^6 293T Lenti-X cells were seeded on 10-
29 cm Petri dishes (Corning Incorporated - Life Sciences, Oneonta, NY, USA) and incubated overnight.
30 Cells were transiently transfected with lentiviral transfer vector expressing influenza HA or NP, along
31 with IN defective packaging and VSV.G-envelope plasmids by Calcium Phosphate using the
32 Profection Mammalian Transfection System (Promega Corporation, Madison, WI) as previously
33 described (13, 398, 410). Plasmids pCAGGS-TMPRSS2 or pCAGGS-HAT and plasmid pCMV3-
34 H1N1-C-NA were included to express protease and NA during IDLV production. To pseudotype
35 IDLV-NP/HA with HA protein, pCMV-HACal09 plasmid was added during IDLV-NP/HA
36 production. After 48 and 72 hrs post-transfection, cell culture supernatants were collected, cleared
37 from cellular debris by low-speed centrifugation and passed through a 0.45- μ m pore size filter
38 (Millipore Corporation, Billerica, MA). To produce IDLV stocks for mouse immunization, vector
39 containing supernatants were concentrated by ultracentrifugation (Beckman Coulter, Fullerton, CA)
40 on a 20% sucrose gradient (Sigma Chemical Co., St. Louis, MO) at 23.000 rpm for 2.5 hrs at 4°C
41 using a SW28 swinging bucket rotor (Beckman). Pelleted vector particles were resuspended in 1X
42 phosphate-buffered saline (Gibco, Life Technologies Italia, Monza, Italy) and stored at -80°C until
43 use. Each IDLV-HA/HA or IDLV-NP/HA stock was titered by the reverse transcriptase (RT) activity
44 assay and the corresponding transducing units (TU) were calculated by comparing the RT activity to
45 the one of IDLV-GFP virions with known infectious titers, thus allowing for the determination of
46 their infectious titer units (421).

47
48 **Flow Cytometry and Confocal laser scanning microscopy (CLSM).** 293T Lenti-X cells were
49 plated in 6-well microplates for flow cytometry analysis or seeded in 24-well cluster plates onto 12-
50 mm cover glasses previously treated with L-polylysine (SIGMA) for CLSM. Cells were then
51 transfected by Calcium Phosphate with pCMVHACal09 using the Profection Mammalian

1 Transfection System (Promega). Twenty-four hours following transfection, non-permeabilized cells
2 were stained with antiserum from CB6F1 mice immunized with purified H1N1 subunit vaccine
3 (0.1µg of HA per dose) from influenza strain H1N1 A/California/7/2009 (provided by Novartis
4 Vaccine & Diagnostics Srl, Siena, Italy) in the presence of MF59 adjuvant (provided by Novartis).
5 After 45 minutes cells were washed in PBS 1X and then incubated for 30 minutes with secondary
6 antibody PE Goat anti-mouse IgG (Biolegend, San Diego, CA, USA), for flow cytometry analysis,
7 or Alexa Fluor-488- F(ab')₂ fragments of goat anti-mouse (Molecular Probes, Life Technologies,
8 Carlsbad, CA, USA), for CSLM analysis. For flow cytometry cells were fixed with 1%
9 paraformaldehyde and fluorescence was measured using the FACScalibur (BD Biosciences, Milan,
10 Italy), and data were analyzed using CellQuest (BD Biosciences). For CLSM, cells were fixed with
11 methanol and the coverslips were mounted with Vectashield® antifade mounting medium containing
12 DAPI (Vector Labs, Burlingame, CA, USA) on the microscope slides. CLSM observations were
13 performed on a Leica TCS SP2 AOBs apparatus (Leica Microsystems, Wetzlar, Germany), using
14 excitation spectral laser lines at 405nm and 488nm, using the confocal software (Leica, Wetzlar,
15 Germany) and Photoshop CS5 (Adobe Systems, San Jose, CA, USA). Signals from different
16 fluorescent probes were taken in sequential scanning mode, several fields were analyzed for each
17 labeling condition, and representative results are shown. Images represented a single central optical
18 section taken in the center of each cell nucleus and a 3D reconstruction.

19
20 **Western blot.** To evaluate HA presence on IDLV particles, pellets of IDLV concentrated
21 preparations were resuspended in SDS loading buffer. Lysed virions were separated on 12% SDS
22 polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane (Sartorius
23 Stedim Italy). Filters were saturated for 2 hr with 5% nonfat dry milk (NFDM) in PBST (PBS with
24 0.1% Tween 20) and then incubated with HA antiserum for 1 hr at room temperature followed by
25 incubation for 1 hr at room temperature with an anti-mouse HRP-conjugated IgG (Sigma Aldrich,
26 Milan, Italy). The immunocomplexes were visualized using chemiluminescence ECL detection
27 system (Luminata Forte Western HRP Substrate, Millipore). H1N1 subunit vaccine from
28 A/California/7/2009 virus was used as positive control.

30 **Mice and immunization schedule**

31 **Ethics statement.** Animals were maintained under specific pathogen-free conditions in the animal
32 facilities at the Istituto Superiore di Sanità (ISS) and treated according to European Union guidelines
33 and Italian legislation (Decreto Legislativo 26/2014). All animal studies were authorized by the
34 Italian Ministry of Healthy and reviewed by the Service for Animal Welfare at ISS (Authorization n.
35 314/2015-PR of 30/04/2015). All animals were euthanized by CO₂ inhalation using approved
36 chambers, and efforts were made to minimize suffering and discomfort.

37
38 CB6F1 female mice were purchased from Harlan (Harlan Laboratory, Srl, San Pietro al Natisone,
39 Italy). Six- to eight-week-old CB6F1 mice, four mice per group, were injected once intramuscularly
40 in the thigh with (i) 1×10^7 RT units/mouse of IDLV-HA/HA, (ii) 1×10^7 RT units/mouse of IDLV-
41 NP/HA, (iii) H1N1 A/California/7/2009 (0.1 µg/mouse of HA protein, henceforth referred to as HAp)
42 alone and (iv) 0.1 µg/mouse of HAp in presence of MF59 adjuvant (HAp+MF59). Naïve, non-
43 immunized mice were kept for parallel analysis. All immunized mice were boosted with HAp alone
44 24 weeks after the prime. Antibodies (Abs) were measured in serum at different time points, starting
45 from 2 weeks after the prime and up to 18 weeks after the boost. The cellular immune responses were
46 analyzed at 4, 12 and 24 weeks after the prime in blood samples and in splenocytes at sacrifice (18
47 weeks after the boost).

48 Serum samples were obtained from blood collected from the retro-orbital plexus of mice with glass
49 Pasteur pipettes and stored at -20°C until assayed. Heparin-treated glass Pasteur pipettes were used
50 to collect blood in order to perform IFN γ ELISPOT assay. Leukocytes, obtained after Ammonium
51 Chloride Potassium (ACK) treatment of whole blood, were counted, suspended in RPMI 1640

1 (Gibco) containing 10% fetal bovine serum (FBS) (Lonza, Treviglio, Milan, Italy), 100 units/ml of
2 PSG (Gibco), non-essential aminoacids (Gibco), sodium pyruvate 1 mM (Gibco), HEPES buffer
3 solution 25 mM (Gibco) 50 mM 2-mercaptoethanol (Sigma Chemicals). Splenocytes were prepared
4 by mechanical disruption and passage through cell strainers (BD Biosciences) and resuspended in
5 complete RPMI medium, as previously described (398).

7 IFN γ ELISPOT

8 The IFN γ ELISPOT assay was performed using the BD ELISPOT kit reagents and protocol (BD
9 Biosciences). Briefly, blood or spleen derived cells were seeded at a density of 2.5×10^5 /well in 96
10 well plates and stimulated overnight either with 2 μ g/ml of the H-2Kd restricted Influenza NP₁₄₇₋₁₅₅
11 (TYQRTRALV) epitope or with 10 μ g/ml of concanavalin A (Sigma Chemicals) used as a positive
12 control. A 139 peptide array (15mers with 11 amino acid overlaps) spanning the entire HA from
13 influenza virus A/California/7/09 protein (BEI Resources, Manassas, VA, USA; Catalog No. NR-
14 15433) was distributed in ten pools of 14 peptides each and used to identify the reactive epitopes on
15 splenocytes. Complete medium treated cells were used as negative controls. Spot Forming Cells
16 (SFC) were counted with an ELISPOT reader (A.EL.VIS, Hannover, Germany) and results expressed
17 as number of IFN γ secreting cells (spot forming cells, SFC)/ 10^6 cells. The samples were scored
18 positive when a minimum of 50 spots per 10^6 cells were present and two fold higher than unstimulated
19 sample.

21 Measurement of binding and functional antibodies

22 Sera were tested for the presence of binding Abs by a standard ELISA. Ninety-six well plates (Greiner
23 bio-one, Kremsmünster, Austria) were coated with H1N1 A/California/4/09 subunit vaccine (0.2
24 μ g/well of HA) overnight at 4°C. After washing and blocking, serial dilutions of serum from
25 individual mice were added to wells in duplicate and incubated for 2 hrs at room temperature. The
26 plates were washed and biotin-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL,
27 USA) was added to the wells for 2 hrs at room temperature. The plates were washed before the
28 addition of horse radish peroxidase (HRP)-conjugated streptavidin (AnaSpec, Fremont, CA, USA)
29 for 30 min at room temperature, followed by the 3,3',5,5-tetramethylbenzidine substrate (SurModics
30 BioFX, Edina, MN, USA). Endpoint titers were determined as the reciprocal of the highest dilution
31 giving an absorbance value at least equal to twofold that of background (biological sample from naive
32 mice). For each group of immunization, results were expressed as mean titer with confidence interval.
33 Haemagglutination inhibition (HAI) Abs to A/California/7/09 virus were measured, according to
34 standard procedures (432). Briefly, all sera were treated with receptor-destroying enzyme (RDE,
35 Sigma Aldrich) to remove non-specific inhibitors of haemagglutination. Serial 2-fold dilutions of
36 treated sera were mixed with 4 haemagglutinin units of the A/California/7/09 virus and, after 1 h
37 incubation at room temperature, with 0.5% turkey red blood cells. The HAI titers were expressed as
38 the reciprocal of the highest dilution of serum that inhibited virus-induced haemagglutination.

39 The sera were tested by MDCK cell-based microneutralization (MN) assay (443) to titrate influenza
40 virus-specific neutralizing antibodies. Briefly, all samples were heat-treated at 56°C for 30 min. Two-
41 fold serial dilutions of samples, starting from 1:10 dilution, were performed across the 96-well plates
42 and then the viral working solution of A/California/07/2009 (H1N1) live virus was added (200
43 TCID₅₀/100 μ l). After incubation of mixture sera-virus at 37°C for 1 h, a cell suspension of 2×10^5
44 was added and the plates were incubated at 37°C for 5 days. Each plate was then checked under an
45 optical microscope to assess the presence of local lesions and cytopathogenic effect (CPE). The
46 neutralization titer (NT) for each serum was calculated according to the Spearman-Kärber formula.

47 Since NA plasmid was included during the IDLV preparation and NA protein may be present in
48 subunit vaccine preparations, the heat-treated sera were also tested by Enzyme-Linked Lectin Assay
49 (ELLA) (454) to measure the functional anti-neuraminidase (anti-NA) antibodies Abs. Briefly, serial
50 2-fold dilutions of treated sera were mixed with antigen A/California/07/2009 (H1N1) treated with
51 Triton X-100 as described elsewhere, in 96-well plates coated with fetuin. The plates were incubated

1 at 37°C overnight (16-18h). After washing the plates, the peanut agglutinin conjugate with peroxidase
2 (PNA-HRPO) was added and the plates were incubated at room temperature for 2h in the dark. Then
3 the o-phenylenediamine dihydrochloride (OPD) substrate was added and the plates were incubated at
4 room temperature for 10 min in the dark. The reaction was stopped by adding 1N H₂SO₄. The Optical
5 Density (OD) of all the test plates was read at 490 nm. The NA inhibition (NI) titer was expressed as
6 the reciprocal of the last dilution that results in at least 50% inhibition of the maximum signal.

7

8 **Statistical analysis**

9 The temporal trend of antibody response (i.e, ELISA, HAI, MN and ELLA) was analyzed using a
10 system of piecewise linear equations in order to jointly evaluate the relationships of each outcome at
11 each time point, allowing for correlated errors. The analysis was conducted in STATA13 (StataCorp
12 LP, College Station, TX) within a structural equation modelling (SEM) frame work (465, 476). The
13 log likelihood estimation procedure was used to fit the model to the data.

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In review

1 RESULTS

2 3 **Production of Integrase Defective Lentiviral Vector (IDLV) delivering Influenza HA**

4 To produce IDLV expressing HA, 293T LentiX cells were transfected with IN defective packaging
5 plasmid, expressing the proteins required for IDLV assembly and release, the pseudotyping VSV.G-
6 expressing plasmid, essential for IDLV entry into target cells, and HA-expressing lentiviral transfer
7 vector plasmid, with the aim of pseudotyping the recombinant IDLV particles with the membrane
8 tethered HA. Importantly, the presence of HA protein on the surface of the transfected cells was
9 confirmed by flow cytometry and confocal microscopy (Fig. 1A and 1B). However, the recovery of
10 IDLV in the supernatant of the transfected cells was very low, indicating inhibition of release of IDLV
11 pseudotyped with HA envelope (Fig. 2A, left column). This was expected since, as with wild type
12 influenza virus, Neuraminidase (NA) protein is required for release of lentiviral vectors pseudotyped
13 with influenza HA (17, 18, 487). To improve production of HA-pseudotyped IDLV, we co-
14 transfected plasmids expressing NA protein, required for cleavage of surface sialic acid molecules on
15 producer cells allowing release of the vector in the supernatant, and human serine transmembrane
16 protease TMPRSS2 or human airway trypsin (HAT) proteins, mediating proteolytic activation of
17 influenza HA (18).

18
19 As shown in Figure 2A, recovery of IDLV-HA increased an average of 8-fold in presence of
20 TMPRSS2 but only 2-fold in the presence of HAT. Importantly, NA was fundamental for IDLV-HA
21 release, increasing the amount of IDLV recovered in the supernatant an average of 24-fold over the
22 IDLV produced in the absence of NA expressing plasmid. The inclusion of TMPRSS2 or HAT in
23 addition to NA protein further improved, although not significantly, vector production (27-fold- and
24 25-fold, respectively, compared to IDLV produced in the absence of NA and proteases). For
25 producing concentrated IDLV preparations for immunization, TMPRSS2 was chosen over HAT
26 protease expressing plasmid since IDLV production was generally higher and more consistent.

27 To produce IDLV expressing NP, vector was produced as described above and in the Materials and
28 Methods section, by including a NP-expressing lentiviral transfer vector plasmid and a plasmid
29 expressing the HA protein for pseudotyping the IDLV produced in the 293T cells. In Figure 2B is
30 shown a representative western blot (WB) analysis of lysates from concentrated stocks of IDLV
31 showing the presence of HA protein in all purified IDLV preparations.

32 33 **A single immunization with IDLV induces high and persistent levels of binding and antiviral 34 neutralizing antibodies**

35 To mimic the immunization protocol of the seasonal influenza in humans, mice were given a single
36 immunization and the immune responses were analyzed at various time points up to 24 weeks. As
37 positive controls, groups of mice were immunized with H1N1 subunit vaccine alone (HAp) or in
38 combination with MF59 (HAp+MF59). The schedule of immunization is described in Figure 3A.

39 Humoral response to H1N1 was assessed initially by ELISA. As shown in Figure 3B, 2 weeks after
40 the prime all vaccinated animals developed anti-H1N1 IgG antibodies in serum. The titers increased
41 and persisted in all groups starting from 8 weeks after immunization. The highest titers were
42 recovered in animals vaccinated with MF59, while the lowest levels were detected in the HAp group.
43 No significant difference was observed between HAp+MF59 group and IDLV groups. Importantly,
44 in both groups of IDLV vaccinated animals sustained Abs were induced at significantly higher titers
45 than HAp alone ($p < 0.01$).

46
47 To assess the recall response, mice were boosted with HAp alone 6 months after the prime. Two
48 weeks after the immunization with HAp all groups showed a boost in terms of binding Ab titers (Fig.
49 3B). In particular, IDLV vaccinated animals were able to increase the anti-H1N1 Abs when boosted
50 with HAp, indicating that immunization with IDLV enables the animals to respond to the HA antigen
51 delivered in a different way, as a subunit vaccine in this case. The responses were persistent up to 18

1 weeks after the boost in all vaccinated animals. After the boost the differences between IDLV
2 immunized animals and HAp group remained statistically significant ($p < 0.01$). Again, no significant
3 difference in Ab titers between both IDLV groups and the adjuvanted group was seen.
4

5 To assess the functional antibodies, haemagglutination inhibition (HAI) assay and
6 microneutralization (MN) assay were performed at 8 and 19 weeks post priming and 2 weeks and 18
7 weeks post boost (Fig. 4). HAI titers were present in all groups at 8 weeks post-immunization and
8 were still detectable at 19 weeks post priming (Fig. 4A). The response significantly increased after
9 the boost in all groups. The kinetics of HAI titers mirrored the kinetics of binding Abs, showing the
10 highest titers in the adjuvant treated group and the weakest ones in the HAp immunized animals. Both
11 groups of IDLV vaccinated animals showed similar levels of HAI titers ($p > 0.05$), significantly lower
12 than HAp+MF59 ($p < 0.05$), but significantly higher compared to HAp vaccinated animals ($p < 0.01$).
13 Interestingly, the MN assay showed absence of neutralizing activity in the HAp alone group of mice
14 after the prime, while MN titers were always present in both groups of IDLV vaccinated animals (Fig
15 4B). Again the highest activity was present in serum samples from HAp+MF59 immunized group.
16 The boost increased the MN titers in all groups, including the HAp immunized animals.
17

18 **IDLV induces anti-NA response**

19 Since NA plasmid was included during the IDLV preparation and NA protein is present in subunit
20 vaccine preparations (498, 5049), we investigated the anti-NA response in all groups of immunized
21 animals. Serum samples were thus assayed for neuraminidase inhibition (NI) activity. As shown in
22 Figure 5, both IDLV groups showed a strong NI activity at all indicated time points after the prime
23 that was significantly boosted after the immunization with HAp. A similar response was detected in
24 the adjuvant vaccinated group, while the animals vaccinated with HAp alone did not generate
25 detectable anti-NA response after the prime, showing low NI activity only after the boost.
26

27 **IDLV vaccination induces T cell response to HA and NP delivered as transgenes**

28 In order to assess the NP specific CD8-restricted cellular response in mice immunized with IDLV-
29 NP/HA, $IFN\gamma$ ELISPOT was performed using blood cells collected at 4, 12 and 24 weeks after the
30 prime, stimulated with MHC Class I-restricted NP peptide (Fig. 6A). As expected, a high number of
31 $IFN\gamma$ producing T cells was detected in animals vaccinated with IDLV-NP/HA overtime, confirming
32 a strong and persistent CD8+ T cell response directed to the transgene delivered by IDLV. The NP-
33 specific T cell response was also analyzed in splenocytes at 42 weeks after a single IDLV-NP/HA
34 immunization further confirming the persistence of transgene-specific $IFN\gamma$ producing CD8 T cells
35 (Fig. 6A).
36

37 In order to evaluate the HA specific response, $IFN\gamma$ ELISPOT was also performed in splenocytes
38 from all groups of mice at sacrifice 18 weeks after the boost (42 weeks after the prime). Ten pools of
39 15mer peptides spanning the entire HA protein were used and the cumulative mean response against
40 the HA pools is shown in Figure 6B. HA-specific $IFN\gamma$ producing cells were detected only in mice
41 primed with IDLV-HA/HA, while no positive response was observed in mice immunized with HAp
42 with or without the adjuvant. In particular, pool 1, pool 9 and pool 10 (mean of 165.4, 666.2, 158.1
43 SFC/ 10^6 cells, respectively) were responsible for the HA-specific response.
44

1 DISCUSSION

2
3 In this study, we assessed the feasibility of improving the strength of immune response for influenza
4 vaccination by administering IDLV engineered to express either HA or NP proteins as transgenes,
5 for induction of T cell responses and to carry HA on the surface of IDLV particles for a concomitant
6 induction of functional antibodies against influenza virus.

7
8
9 Immunization with either HA-pseudotyped IDLV induced functional and durable Ab responses that
10 were further increased after boosting with H1N1 purified subunit vaccine. This suggests that for
11 induction of HA-specific Abs, HA protein does not need to be expressed from the IDLV transgene.
12 Titers were lower or comparable to those obtained after immunization with MF59-adjuvanted H1N1
13 purified subunit vaccine, which we used as a gold standard for the induction of a strong and effective
14 antibody response against influenza virus in mice. In particular, persistent HAI titers and anti-HA
15 neutralizing Abs against homologous influenza virus strain were detected throughout the time course
16 after the prime and were further increased after the boost. Of note, mice immunized with both IDLV-
17 HA/HA and IDLV-NP/HA induced functional anti-NA Abs, which were detected after the prime and
18 increased after the boost, as measured by the NI assay. Titers were comparable to those obtained after
19 immunization with MF59-adjuvanted H1N1 purified subunit vaccine, but significantly higher
20 compared to HAp immunized animals. NA activity is required for the release of HA-pseudotypes of
21 IDLV from producer cells and a plasmid expressing the NA protein was included during preparation
22 of IDLV for enabling vector production, as described in other settings (18). Although NA-specific
23 Abs may not effectively prevent viral infection in humans, they may inhibit virus spread and reduce
24 the severity of disease (510, 524), and a recent report provided strong evidence that NI titers correlated
25 more significantly with reduced disease severity in a healthy volunteer challenge study performed
26 with a wild-type influenza A challenge virus (532). Additional characterization of IDLV vaccine-
27 induced functional antibodies, such as mapping of broadly neutralizing antibodies directed to the stem
28 region of HA, will provide further information on the value of our platform.

29
30 While induction of HA-specific antibodies represents the primary strategy for prevention and control
31 of influenza after vaccination in humans (3653), ~~CD8+ T cell responses against influenza virus~~
32 ~~proteins are often induced and may contribute to heterosubtypic protection (54-56).~~ The highly
33 conserved viral NP has become an important focus for the development of broad, cross-protective or
34 “universal” influenza vaccines. NP specific cell-mediated responses have been shown to be protective
35 against homologous and heterosubtypic influenza virus challenge in animal models (13, 54-57) and
36 may help in reducing disease severity through enhancing viral clearance in humans (30, 58). ~~For this~~
37 ~~reason, we also evaluated T cell response induced by IDLV over time.~~ In our previous work (13) we
38 assessed the ability of IDLV-NP to induce protective immunity using different routes of
39 immunization. In particular, we demonstrated that intranasal administration of IDLV-NP was more
40 efficient than intramuscular immunization in protecting mice from influenza virus challenge.
41 Although the protection from influenza challenge in mice can be achieved in presence of NP-specific
42 T cell responses, this correlation has not been confirmed in humans, where the T cell responses
43 directed toward conserved proteins may instead help in controlling the disease symptoms (28-30). To
44 further improve the strength of our vaccine and to render the platform more suitable for human use,
45 in the present work we focused on the development of a new vector strategy for expressing influenza
46 antigens not only as transgenes but also as surface molecules. Here we demonstrated that the multi
47 antigen IDLV-based influenza vaccine induced durable and functional immune responses in terms of
48 NP or HA transgene-specific T cell responses and protective antibodies directed towards the surface
49 proteins HA and NA. Overall our results indicate that immunization with IDLV HA/HA and IDLV-
50 NP/HA induced cell mediated immune responses against the respective transgene. In particular, NP-
51 specific CD8-restricted cellular responses, as measured by INF γ ELISPOT, were present and

1 maintained up to 42 weeks after a single immunization in mice immunized with IDLV-NP/HA.
2 Similarly, HA-specific IFN γ producing cells were detected in mice immunized with IDLV-HA/HA,
3 but not in mice immunized with HAp with or without MF59 adjuvant.
4

5 Other approaches have been tested for delivering multiple antigens for inducing cellular and humoral
6 immunity. As an example, a recent report using Adenovirus and MVA delivering Influenza NP, M1
7 and HA antigens in a prime-boost regimen showed induction of immune responses against the
8 delivered antigens and protection after challenge (59). The approach described in our report shows
9 that multivalent IDLV efficiently induce a prolonged cellular immune response due to expression of
10 the vectored HA and NP transgenes from episomal DNA circles, as described in several settings (1,
11 2), and functional humoral responses to HA and NA proteins.
12

13 Previous work has shown that recombinant influenza vaccines containing insect cell-expressed virus-
14 like particles (VLPs) displaying H1N1 may constitute a promising vaccination approach (60).
15 Pseudotype-based influenza genes delivery represents an alternative to successfully express HA in
16 mammalian cells providing an efficacious vaccine when tested in chickens and mice (61, 62). More
17 recently, Venereo-Sanchez and co-workers showed that HIV-Gag based VLP displaying H1N1
18 induced a sustained immune response which provided full protection after lethal challenge with the
19 homologous virus strain in mice (63). Of note, IDLVs used in our report were pseudotyped with
20 VSV.G glycoprotein which may also contribute to the strength of the IDLV-induced immune
21 responses by increasing the tropism of the vector. In fact, it has been shown that HIV virus-like
22 particles (VLP) pseudotyped with VSV-G are more immunogenic compared to VLP that lacked VSV-
23 G, in a monkey model of immunization (64) and that VSV-G-pseudotyped LV can adhere to
24 transduced cells for a substantial amount of time, thus leading to additional cycles of transduction
25 (65-67). These are potentially important advantages for exploiting recombinant IDLV pseudotyped
26 with VSV.G and/or other heterologous viral proteins.
27

28 In conclusion, our study highlights the potential for IDLV to be developed for use as a novel multi-
29 antigen vaccine platform against influenza virus. Combination of NP, HA and NA antigens in the
30 same IDLV results in a more comprehensive and functional immune response, which may be
31 beneficial to prevent and/or control influenza virus infection.
32

1 **Author Contributions**

2 AG, ACara and DN designed the experiments, analyzed the data and wrote the paper; AG, MB, RB,
3 FG, LC, LP, SC, DM, VM, AC and ZM performed experiments; MFV performed statistical analysis;
4 MRC analyzed the data and critically edited the manuscript; NT provided technical knowhow on
5 pseudotype production and critically edited the manuscript; MS provided key reagents and critically
6 edited the manuscript. All authors have contributed to the drafting of the manuscript, have revised
7 the work and have approved the final version.

8
9 **Conflict of Interest Statement**

10 The authors declare that the research was conducted in the absence of any commercial or financial
11 relationships that could be interpreted as a potential conflict of interest.

12
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25

FIGURES LEGENDS

Figure 1. Analysis of HA expression. 293T Lenti-X cells were transfected with HA expressing plasmid and stained at 24 hours post-transfection for detection of HA on the plasma membrane as described in Materials and Methods. Cells were fixed and expression of HA was quantitatively measured by flow cytometry (A) or observed by CLSM (B). (A) The percentage of HA-expressing cells is indicated. The overlay line (green) represents the fluorescence distribution of cells stained only with the secondary Ab. (B) Images represent single central optical sections (a-c) and a 3D reconstruction (d). Nuclei are colored in blue by DAPI staining and green color represents membrane associate HA protein. Scale bars, 8 μ m. Untransfected cells (a) were used as negative control. Results from one representative experiment are shown for each analysis.

Figure 2. Production and validation of IDLV pseudotyped with HA. (A) Reverse transcriptase (RT) activity of IDLV expressing HA. Vectors were produced as described in Materials and Methods and in the presence of plasmids expressing TMPRSS2, HAT or NACal09, as indicated in the graph. Data are expressed as the mean result from three independent experiments. The error bars represent the standard errors of the mean. (B) Western blot (WB) of lysates from concentrated stocks of IDLV pseudotyped with HA showing incorporation of HA into IDLV. Note that IDLVs were produced in the presence of TMPRSS2 protease, resulting in the cleavage of HA0 to produce HA1 (not visualized here) and HA2. HA protein (HAp*, purified hemagglutinin vaccine subunit from influenza virus H1N1 A/California/7/2009) and IDLV-GFP were used as positive and negative control respectively.

Figure 3. Immunization schedule and kinetics of anti-H1N1 binding Abs. (A) Immunization schedule. CB6F1 mice (4 mice/group) were primed once intramuscularly with IDLV expressing HA and pseudotyped with HA (IDLV- HA/HA), IDLV expressing NP and pseudotyped with HA (IDLV- NP/HA), HA protein (*purified hemagglutinin vaccine subunit from influenza strain H1N1 A/California/7/2009) in combination with MF59 as an adjuvant (HAp+MF59), HA protein alone (HAp) or left untreated (Naive). All groups except for naive were boosted with HAp at 24 weeks after the prime. Blood was collected at several time points in order to perform ELISA and IFN γ ELISPOT assays. (B) Kinetics of serum anti-H1N1 IgG Abs. Serum samples from all groups were collected at the indicated time points after the prime and were assayed for the presence of anti-H1N1 IgG by ELISA. Results are expressed as predicted mean endpoint titers. The predicted mean values at each time point were estimated by a system of piecewise linear regressions including all antibody measurements within a structural equation modelling (SEM) frame work. Error bars indicate the 95% confidence interval. Asterisks indicate significant differences between groups at all the analyzed time points; ** p value<0.01.

Figure 4. Kinetics of functional anti-HA Abs. Serum samples from all groups were collected at the indicated time points after the prime and were assayed for the presence of neutralizing Abs against A/California/7/09 virus, by HAI assay (A) and MN assay (B). Results are expressed as predicted mean endpoint titers. The predicted mean values at each time point were estimated by a system of piecewise linear regressions including all antibody measurements within a structural equation modelling (SEM) frame work. Error bars indicate the 95% confidence interval. Asterisks indicate significant differences compared to the HAp group, at the indicated time points; **p<0.01; *p<0.05.

Figure 5. Kinetics of functional anti-NA Abs. Serum samples from all groups were collected at the indicated time points after the prime and were assayed for the presence of anti-NA Abs by ELLA assay (see Materials and Methods). Results are expressed as predicted mean endpoint titers. The predicted mean values at each time point were estimated by a system of piecewise linear regressions including all antibody measurements within a structural equation modelling (SEM) frame work. Error

1 bars indicate the 95% confidence interval. Asterisks indicate significant differences compared to the
2 HAp group at all the analyzed time points; ** p value<0.01.

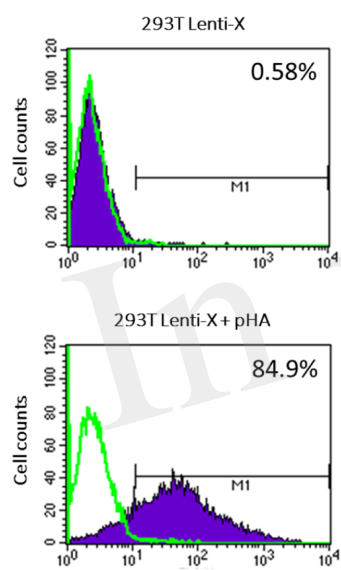
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4 **Figure 6. Analysis of antigen-specific T cells response.** (A) Kinetics of NP-specific T cell response
5 in mice immunized with IDLV-NP/HA. IFN γ ELISPOT was performed using blood cells collected
6 at 4, 12 and 24 weeks post prime and splenocytes collected at 42 weeks post prime. Results are
7 expressed as mean spot forming cells (SFC) per 10⁶ cells. Cells were stimulated overnight with the
8 MHC I-restricted epitope derived from NP protein sequence (black bars) or left untreated (white bars).
9 Error bars indicate the standard deviation among mice of the same group. (B) Analysis of HA-specific
10 T cell response in immunized mice. IFN γ ELISPOT was performed using splenocytes collected at 42
11 weeks post prime, using 10 pools of 15mers spanning the full length of HA protein, as described in
12 Materials and Methods. Results are expressed as cumulative mean spot forming cells (SFC) per 10⁶
13 cells for each pool among mice of the same indicated group.

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In review

Figure 1.TIF

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B

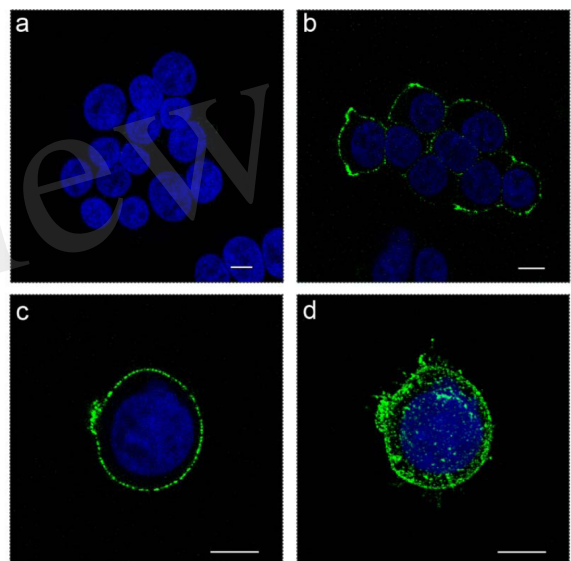
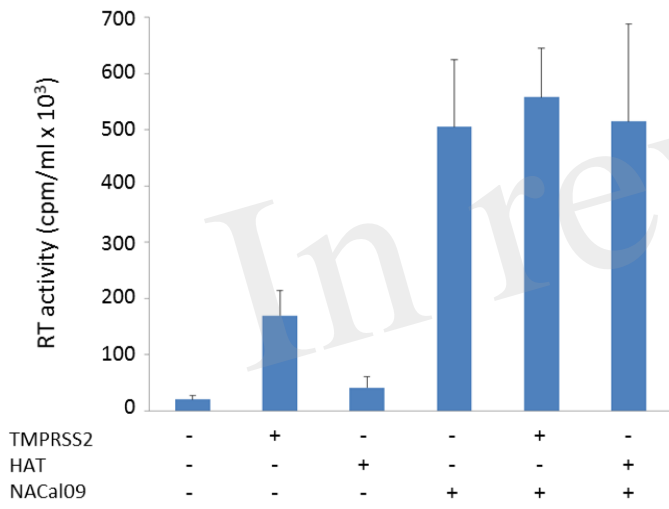


Figure 1

Figure 2.TIF

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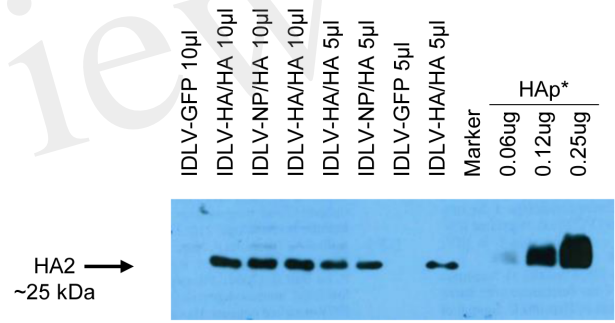
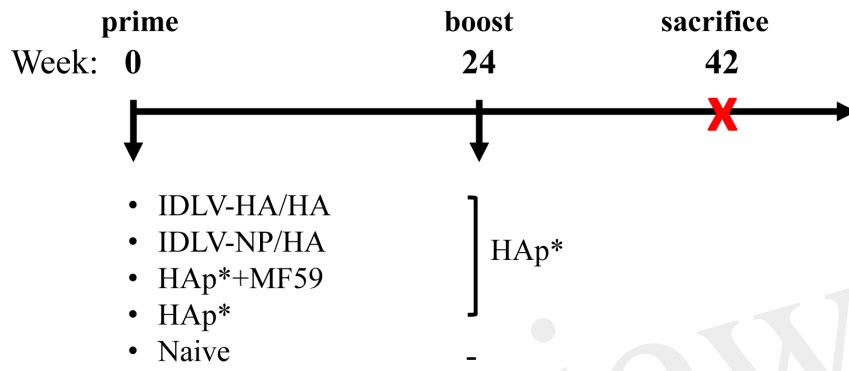


Figure 2

Figure 3.TIF

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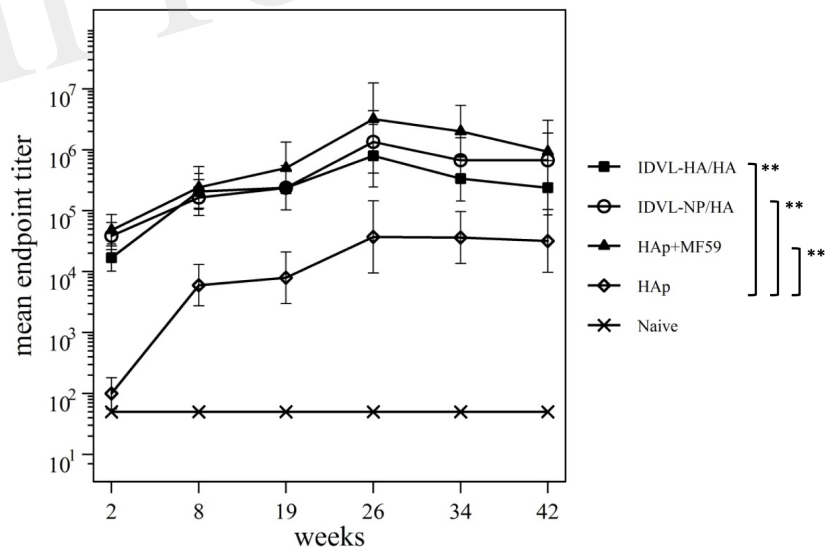
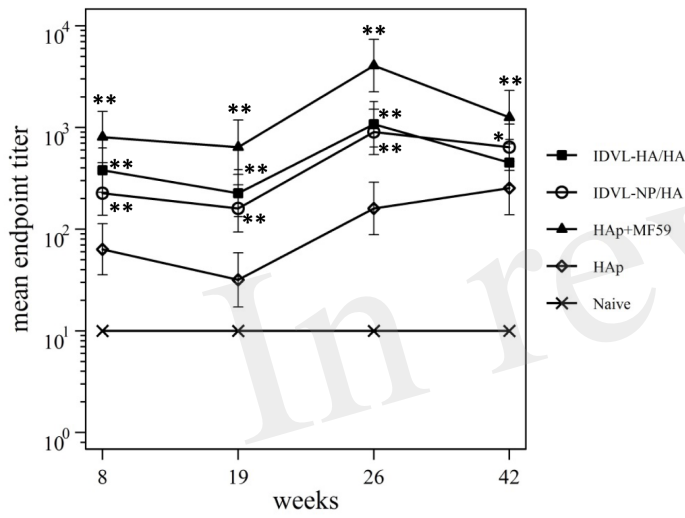


Figure 3

Figure 4.TIF

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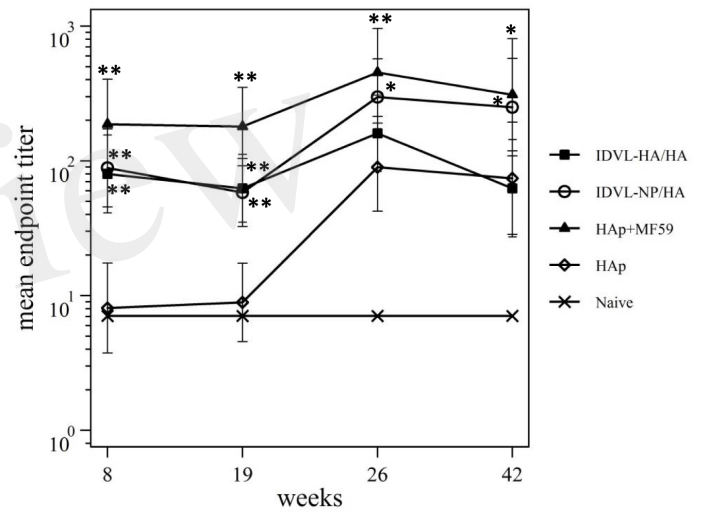


Figure 4

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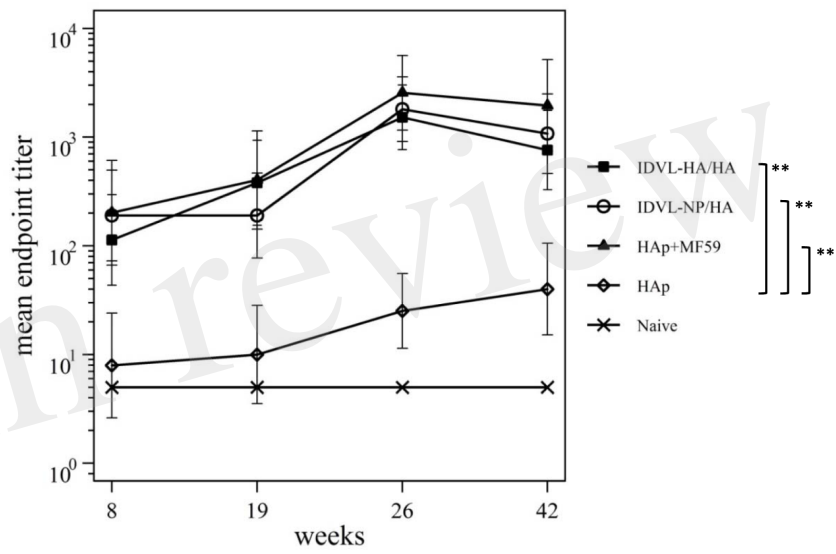


Figure 5

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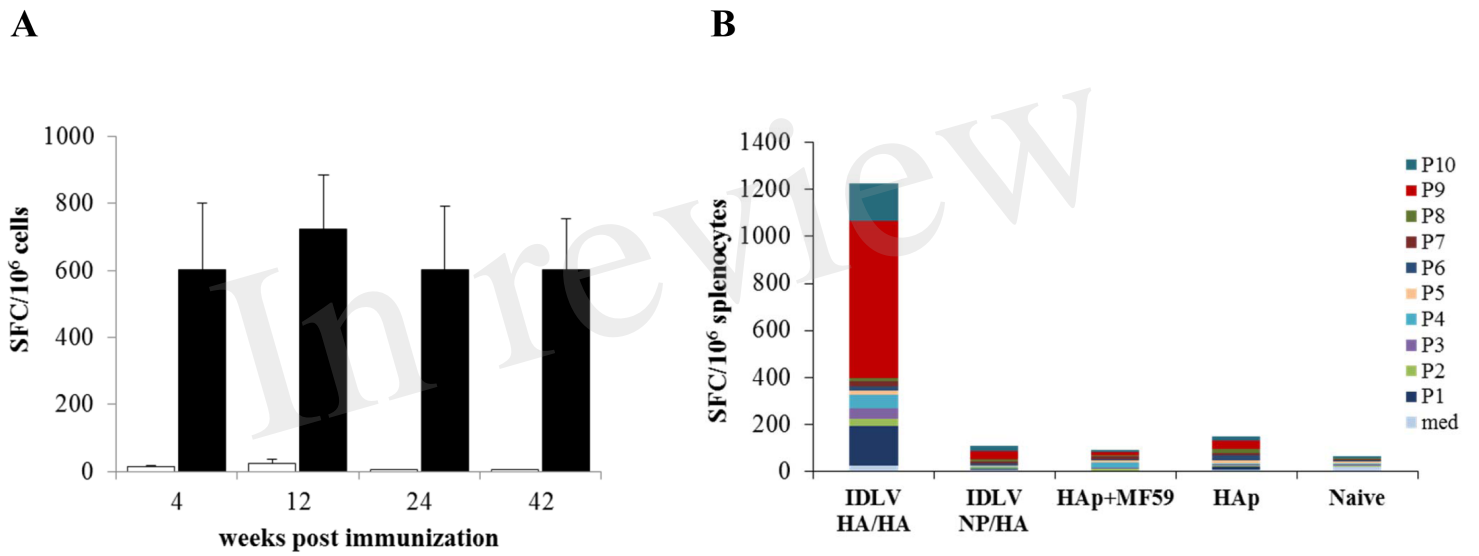


Figure 6