Short communication

Targeting of HA to chemokine receptors induces strong and cross-reactive T cell responses after DNA vaccination in pigs

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1. Introduction

Conventional influenza vaccines are hampered by a prolonged production time, with a major consequence being reduced vaccine efficacy [1]. The continuous antigenic drift of circulating influenza strains makes it difficult to decide specifically which strains should be included in the vaccine formulation, and the longer the period needed between decision and vaccine deployment, the more inaccurate the selection is likely to be.

DNA vaccines allow for rapid production and vaccine deployment, and can as such ensure better matching of vaccine strains to circulating strains. However, a challenge with DNA vaccines is that although they can induce strong immune responses in mice, they generally fail the translation to larger animals and humans that bind chemokine receptors 1, 3, and 5 expressed on antigen presenting cells (APC). Such MIP1α targeting of HA to APC enhanced induction of HA reactive antibodies, particularly IgG2. In addition, the MIP1α- HA vaccine induced strong T cell responses that could cross-react with different influenza subtypes. Thus, the strategy of targeting HA to chemokine receptors could be important for inducing broad protection against antigenically diverse influenza strains in pigs.

Keywords: DNA vaccine, Influenza, Chemokine receptor, APC-targeting, Pig

ABSTRACT

Efficient influenza vaccination of pigs can reduce disease burdens for the swine industry, but also represents an important measure for reducing the risk from novel viral reassortments that pose pandemic threats to the human population. Here, we have vaccinated pigs with a DNA vaccine encoding influenza virus hemagglutinin (HA) linked to the chemokine MIP1α that bind chemokine receptors 1, 3, and 5 expressed on antigen presenting cells (APC). Such MIP1α targeting of HA to APC enhanced induction of HA reactive antibodies, particularly IgG2. In addition, the MIP1α- HA vaccine induced strong T cell responses that could cross-react with different influenza subtypes. Thus, the strategy of targeting HA to chemokine receptors could be important for inducing broad protection against antigenically diverse influenza strains in pigs.

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The HA-specific mAb H36-4-52 was a kind gift from Siegfried Weiss, Braunschweig [13].

2.2. Vaccine construction

The nucleotide sequence for pig MIP1α was obtained from NCBI (accession nr. NM_001009579). Since the vaccine vector already contained the leader sequence from human IgG3 [14], the endogenous signaling peptide (aa 1–23) was removed. Next, the chemokine was cloned on BsmI/HindIII-sites into a previously described vaccine format equipped with HA from influenza A/PuertoRico/8/1934 (H1N1) (PR8) [4] (Fig. 1A).

2.3. Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of vaccine proteins

ELISA plates (Costar 3590, Sigma-Aldrich, St. Louis, MO, US) were coated with mAb MCA878 (anti-human CH3) (AbD Serotec, CA, US), blocked, and incubated with supernatants from 293E-cells transiently transfected with the different vaccine plasmids. Next, plates were incubated with biotinylated H36-4-52, and then streptavidin alkaline phosphatase (GE Healthcare, Buckinghamshire, UK). Plates were developed using phosphatase substrate (P4744-10G, Sigma Aldrich) dissolved in substrate buffer, and read with a Tecan reader (Tecan, Switzerland) using the Magellan v5.03 program.

2.4. Chemotaxis

The vaccine plasmids were transiently transfected into 293E cells, and chemotactic integrity assessed by quantifying ESb-MP [15] cell migration across a 5 μm pore polycarbonate membrane in response to the titrated presence of vaccine proteins or a positive control (recombinant LD78β, Peprotech), as previously described [16]. Results from triplicate samples (mean) are presented as chemotactic index, defined as the fold increase of cells migrating in the presence of chemotactic factors over spontaneous cell migration (i.e. in medium alone).

2.5. Vaccine binding to pig peripheral blood mononuclear cells (PBMC)

Pig PBMC were collected by Lymphoprep separation (Axis-Shield, Oslo, Norway) of fresh whole blood, and stained with vaccine proteins in supernatants from transiently transfected 293E cells. Next, the cells were incubated with phycoerythrin (PE)-conjugated mAbs against human IgG (2043-09, SouthernBiotech, P4744-10G, Sigma Aldrich) dissolved in substrate buffer, and read with a Tecan reader (Tecan, Switzerland) using the Magellan v5.03 program.
AL, US) to detect vaccine proteins, fluorescein isothiocyanate (FITC)-conjugated mAb against CD3ε (559582, BD Biosciences, NJ, US), and mAb against CD11R3 (MCA2309, AbD serotec, CA, US) [detected with allophycocyanin (APC)-conjugated mAb against IgG1 (550874, BD Pharmingen, CA, US)]. Cells were run on a BD FACScanibur system (BD Biosciences, NJ, US) and analyzed by FlowJo software (Version 7.6) (FlowJo, OR, US).

2.6. Animals and vaccination

Six- to eight week old pigs of both sexes were used (Noroc: 50% Norwegian landgris, 25% Norwegian Yorkshire and 25% Duroc). Weights at the start of the experiments ranged from 16 to 30 kg, whereas weights at the termination of experiments ranged from 34 to 48 kg. Pigs were housed at the Animal Production Experimental Centre, Norwegian University of Life Science, Ås, Norway. Experiments were approved by the Norwegian Animal Research Authority (Oslo, Norway), in accordance with the EU Directive 2010/63/EU. Pigs were confirmed negative for influenza reactive antibodies (<23) in HI-assays against influenza A/California/7/2009 (H1N1) (Cal07) and PR8, and immunized by intradermal (i.d.) jet delivery (Tropis, PharmaJet, DE, US), and mAb against CD11R3 (MCA2309, AbD serotec, CA, US) [deposited in the Phox library (accession nr. NM_001009579), and inserted into a previously described dimerization unit consisting of the hinge and CH3 from human IgG3 that conferred bivalent display of antigens and targeting activity of pig MIP1α]. Further, the MIP1α property of pig MIP1α was found to be intact (Fig. 1D). Furthermore, the MIP1α-containing vaccine proteins bound pig PBMC, in contrast to non-targeted vaccine proteins, confirming that the chemokine was functional in our vaccine (Fig. 1E).

2.7. Serum ELISA

Sera were isolated from blood by centrifugation. ELISA 96-well plates (Costar 3590, Sigma-Aldrich) were coated with either recombinant HA from PR8 (11684-V08H, Sino Biological Inc., North Wales, PA, US), rec. HA from A/Anhui/1/2005 (H5N1) (11048-V08H1, Sino Biological Inc.), rec. HA from A/Shanghai/1/2013 (H7N9) (40104-V08H, Sino Biological Inc.), or the HA stem domain linked to a Phox-specific scFv on a coat of Phox-bovine serum albumin (BSA). Plates were now blocked with 0.1% BSA in PBS, and incubated overnight at 4 °C with titrated amounts of sera. HA-specific antibodies were detected with either biotinylated anti-pig IgG (ab112747, Abcam, Cambridge, UK), or anti-pig IgG1 (MCA635GA, Bio-Rad Laboratories, Hercules, CA, US), or anti-pig IgG2 (MCA636GA, Bio-Rad Laboratories). The two latter were then incubated with biotinylated anti-mouse Fc (079K4788, Sigma-Aldrich). Next, samples were incubated with streptavidin alkaline phosphatase (GE Healthcare). Plates were analyzed with the CTL-ImmunoSpot vector [17] (kind gift from Bob Weinberg, Addgene plasmid #8449) on days 0 and 28.

2.8. Microneutralization assay for influenza virus-specific antibodies

The microneutralization assay was performed as previously described [4]. Briefly, sera were treated with receptor destroying enzyme (RDE) (II) (Denka Seiken, Tokyo, Japan), and twofold dilutions set up in triplicates. Fifty μl of 100x tissue culture infective dose (TCID₅₀) virus PR8 was added to each well, and plates incubated for 2 h at 37 °C in a 5% CO₂ humidified atmosphere. Madin-Darby canine cells (MDCK) cells (2×10⁵) were added to each well, and plates incubated for 20 h at 37 °C and 5% CO₂. Monolayers were washed with PBS and fixed in cold 80% acetone for 10 min, and viral proteins detected by an ELISA using biotinylated mAb against the influenza nucleoprotein (HB665, ATCC, VA, US) and streptavidin-alkaline phosphatase (GE Healthcare). Plates were read as described above, and the % neutralization of each serum sample calculated with the following equation: x = [(average OD of VC wells)-(OD of sample)]/[(average OD of VC wells)-(average OD of CC wells)] × 100.

2.9. Hemagglutination-inhibition (HI)-assay

Sera were pre-treated with RDE (II) (Denka Seiken), and titrated in triplicates across 96-well plates. Following a 40 min incubation with PR8 virus (4HAU), Turkey red blood cells (1%) were added, and plates read 45 min later. HI was scored as the highest dilution of antiserum giving a complete inhibition of hemagglutination. Validity of results were confirmed by a positive control serum (influenza PR8 antiserum, Charles River) reaching its predicted titer, and a negative control serum giving a titer of < 2³.

2.10. Enzyme-linked immune absorbent spot (ELISpot) assay

ELISpot assay was performed as described in the kit protocol for ELISpotPLUS for Porcine IFN-γ (3130-2AW-Plus, Bio-Rad). Briefly, PBMC that previously had been separated from whole blood by Leucosep separation (163290, Greiner Bio-one) and frozen in liquid nitrogen, were thawed and rested over night at 37 °C in 5% CO₂. Next, cells were plated out in 5×10⁵ cells/well, and stimulated for 20 h at 37 °C in 5% CO₂ with either rec. HA from influenza PR8 (10 μg/ml), Concanavalin A (5 μg/ml), HA from A/Anhui/1/2005 (H5N1) (11048-V08H1, Sino Biological Inc.), or HA from black-headed gull/Netherlands/1/2000 (H13N8) (11721-V08B, Sino Biological Inc.), ovalbumin, or medium alone. Next, plates were incubated with the detection antibody (3130-2AW-Plus, Bio-Rad), and developed with the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/ nitro blue tetrazolium (NBT)-purple liquid substrate system for membranes (B3679, Sigma-Aldrich). Plates were analyzed with the CTL-ImmuNoSpot³ analyzer (CTL, Shaker Heights, OH, USA).

2.11. Statistical analyses

Statistical analyses were performed using analysis of variance (ANOVA) and Bonferroni’s multiple comparison test with the Graphpad Prism software (GraphPad Software Inc. version 5).

3. Results and discussion

3.1. Construction of vaccine targeting antigen to pig chemokine receptors

The sequence for porcine MIP1α was obtained from NCBI (accession nr. NM_001009579), and inserted into a previously described vaccine format [3,4] (Fig. 1A). The vaccine contained a dimerization unit consisting of the hinge and C₃ from human IgG3 that conferred bivalent display of antigens and targeting units. Porcine MIP1α served as targeting units, with HA from influenza A/PuertoRico/8/1934 (H1N1) (PR8) included as antigen (Fig. 1B). A previously described non-targeted vaccine control containing a scFv specific for the hapten 4-hydroxy-3-iodo-5-nitrophenylnitric acid (NIP) was also included [4].

Vaccine proteins were secreted at comparable levels after transient transfections of 293E cells (Fig. 1C), and the chemotactic property of pig MIP1α was found to be intact (Fig. 1D). Furthermore, the MIP1α-containing vaccine proteins bound pig PBMC, in contrast to non-targeted vaccine proteins, confirming that the chemokine was functional in our vaccine (Fig. 1E).
3.2. Antibody induction after DNA vaccination

Norwegian farm pigs were confirmed negative for pre-existing antibodies against HA (HI titer < 23), and DNA vaccinated by i.d. jet delivery. A single DNA vaccination with MIP1α-HA significantly enhanced levels of IgG in sera, as compared to vaccination with the non-targeted control vaccine αNIP-HA, or HA alone (Fig. 2A). A second vaccination further boosted antibody responses after vaccination with MIP1α-HA, but also increased antibody responses after vaccination with the non-targeted control vaccine αNIP-HA. A closer examination of the induced antibodies showed no significant differences in IgG1 levels between MIP1α-HA and αNIP-HA (Fig. 2B), but targeting of HA to chemokine receptors significantly enhanced serum levels of IgG2 (Fig. 2C). Interestingly, the IgG2 levels remained significantly elevated also after the second vaccination with MIP1α-HA. Porcine IgG2 is one of six different porcine subclasses described [18]. The subclass is associated with the presence of IFNγ, and has the potential to activate complement [19].

For an assessment of potential qualitative differences between the vaccine-induced antibodies, sera were examined in a microneutralization assay. A single vaccination with either MIP1α-HA or αNIP-HA significantly raised the levels of neutralizing antibodies, as compared to vaccination with HA alone (Fig. 2D). The second vaccination further boosted the neutralizing potential after vaccinations with either MIP1α-HA or αNIP-HA, in contrast to vaccination with HA alone (Fig. 2E). An HI-assay against influenza PR8 confirmed these results (Fig. 2F).

Even if the neutralizing capacity was similar between sera in pigs vaccinated with αNIP-HA and MIP1α-HA, the observed differences in subclass distribution could indicate different in vivo functionalities [20,21]. Thus, sera were examined for cross-reactivity against HA from H5 and H7 influenza viruses, and the HA stem

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Fig. 2. Antibody responses after DNA vaccination in pigs. Norwegian farm pigs (n = 6/group) were immunized twice (days 0 and 28) with 75 μg DNA i.d. by Jet delivery. (A-C) Sera obtained at the indicated time points were tested for IgG (A), IgG1 (B), and IgG2 (C) antibodies binding rec. HA (PR8) (mean ± SEM). * = p < 0.05 as compared to αNIP-HA, HA and NaCl; two-way ANOVA. (D, E) Sera from day 28 after a single vaccination (D), or one week after the second vaccination (E) were assayed in micro-neutralization assays against influenza PR8. Individual values are given, and the dotted lines indicate threshold for positive neutralization. * = p < 0.05 as compared to HA and NaCl; one-way ANOVA. (F) Sera from day 28 after a single vaccination and one week after the second vaccination (day 35) were assayed in an HI-assay against influenza PR8. (G-I) Sera were tested for IgG against rec. HA from influenza H7N9 (G), influenza H5N1 (H), or the stem domain of influenza PR8 (I) (mean ± SEM). * = p < 0.05 as compared to αNIP-HA, HA and NaCl; two-way ANOVA.
domain from PR8 HA. While no responses were observed against HA from H7 influenza (Fig. 2G), significant (albeit low) antibody titers were observed against H5 influenza virus after vaccination with MIP1α-HA (Fig. 2H). This result is in line with influenza virus phylogeny, since influenza viruses can be divided into two main groups based on structural similarities particularly in the HA stem region[22]. The H7 influenza viruses belong to group 2, and H1 and H5 to group 1. The fact that antibodies also bound the HA stem domain (Fig. 2I), which is highly conserved among influenza group 1 subtypes, indicates that some cross reactivity could be expected also against other members of group 1. The biological significance of these cross-reactive antibodies is not clear, particularly since the levels are quite low.

Previously, vaccination with conserved influenza antigens in pigs has been demonstrated to be associated with vaccine-associated enhanced respiratory disease (VAERD)[23]. The precise reason for this association is not known, but an association with antibodies against the stem domain of influenza has been suggested[24]. More studies are needed to evaluate the immunological mechanisms behind VAERD, and particularly in the context of universal protection against influenza.

3.3. T cell responses after DNA vaccination

Pigs were DNA immunized i.d. by jet delivery, and PBMCs collected from blood at day 28 after the first vaccination and then seven days after the second vaccination (day 35). In order to examine vaccine induced T cell responses, PBMCs were stimulated with recombinant HA from influenza PR8, and the levels of secreted IFNγ assayed by ELISpot. Interestingly, vaccination with MIP1α-HA significantly raised the numbers of IFNγ-secreting cells after a single vaccination, as compared to vaccination with the non-targeted control vaccine αNIP-HA or HA alone. This increase was also maintained after the second DNA vaccination, and indicated a strong potential for T cell based protection against influenza (Fig. 3).

Fig. 4. Cross-reactive T cell responses after a single DNA vaccination. PBMCs collected from pigs at day 28 after vaccination were examined for cross-reactive T cells against rec. HA from influenza H5 and H13, as well as ovalbumin and medium alone (mean ± SEM). *p < 0.05 as compared to αNIP-HA, HA and NaCl; one-way ANOVA.

T cell epitopes are typically more conserved than B cell epitopes. Thus, the collected PBMCs were also assayed in ELISpot assays after in vitro stimulations with HA from influenza H5 and H13 (Fig. 4). Interestingly, a significant increase of IFNγ-secreting cells was observed after vaccination with MIP1α-HA, but not the non-targeted control vaccines. This result again confirmed that targeting of antigen to chemokine receptors was particularly efficient for raising cellular immune responses in pigs.

4. Conclusion

Previously, targeting of antigen to APC has been demonstrated to greatly increase vaccine efficacy. Here, we found that targeting of influenza virus HA to chemokine receptors enhanced vaccine immunogenicity in pigs, and polarized the induced immune response towards a T cell phenotype. Importantly, the T cell responses cross-reacted against different influenza virus subtypes, and indicated a potential for broad protection against influenza.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [BB is inventor on patent applications filed on the vaccine molecules by the TTO offices of the University of Oslo and Oslo University Hospital, according to institutional rules. BB is head of the Scientific panel of Vaccibody AS, and hold shares in the company].

Acknowledgements

We thank Pharmajet Inc. for providing the apparatus for jet injections. The technical help of Elisabeth Vikse is gratefully acknowledged. Furthermore, we gratefully acknowledge the technical help of Linda Andreassen and the other staff at the Animal Production Experimental Centre, Norwegian University of Life Science, Ås, Norway.

Author contributions

GG and BB conceived and designed experiments. GG and EF performed experiments. GG analysed experiments, and wrote the paper. All authors commented and edited on the paper.
Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.11.084.

References


