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DNA Vaccine that Targets Hemagglutinin to MHC Class II Molecules Rapidly Induces Antibody-Mediated Protection against Influenza

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New influenza A viruses with pandemic potential potentially emerge due to viral genomic reassortment. In the face of pandemic threats, production of conventional egg-based vaccines is time consuming and of limited capacity. We have developed in this study a novel DNA vaccine in which viral hemagglutinin (HA) is bivalently targeted to MHC class II (MHC II) molecules on APCs. Following DNA vaccination, transfected cells secreted vaccine proteins that bound MHC II on APCs and initiated adaptive immune responses. A single DNA immunization induced within 8 d protective levels of strain-specific Abs and also cross-reactive T cells. During the Mexican flu pandemic, a targeted DNA vaccine (HA from A/California/07/2009) was generated within 3 wk after the HA sequences were published online. These results suggest that MHC II–targeted DNA vaccines could play a role in situations of pandemic threats. The vaccine principle should be extendable to other infectious diseases.

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Recombinant protein subunit vaccination is also an unlikely alternative due to limitations in mass production. Hence, it is of utmost importance to develop novel influenza vaccines that can be rapidly produced upon pandemic threats.

DNA vaccines encoding viral subunits can be rapidly generated, and may present the timeline and production efficiency needed to prevent a pandemic. However, the induction of adequate immune responses against influenza require large doses of DNA even in mice, and often in combination with adjuvants or several booster injections (4–10). Also, DNA vaccines often have a tendency of skewing immune responses toward T cell immunity, whereas Abs are of major importance in influenza prevention. Thus, there is a need for novel DNA vaccines against influenza that rapidly can induce protective Abs after a single injection.

The immunogenicity of Ags can be increased by targeting of Ag to various surface molecules on APCs (11–23). Such targeting has typically been done to obtain increased MHC presentation of Ag and thus increased T cell responses (20, 21, 23). However, in some studies, APC targeting resulted in increased Abs responses (11–14). Notably, several reports have demonstrated that immunization with anti–MHC class II (MHC II) mAb chemically conjugated to avidin (12, 19), FITC (16), and hemagglutinin (HA) (17) enhanced Abs responses. However, Ab–Ag complexes are difficult to produce and thus increase batch-to-batch variability (24). Use of Ab–Ag conjugates is thus not a plausible scenario for mass vaccination. Recombinant fusion proteins, with high batch-to-batch consistency, could be an alternative, but suffer from difficulties in mass production.

Perhaps solving the above problems, we (25–27) and others (20) have previously shown that DNA vaccination and APC targeting can be combined. Thus, DNA that encodes fusion proteins targeting Ags to MHC II molecules (25), CD40 (27), and chemokine receptors (20, 26) increased Ab and T cell responses in mice against idiotypic tumor-specific Ag and protection against a subsequent tumor challenge (20, 25–27). It was further demonstrated that DNA-injected and electroporated muscle cells secreted fusion proteins that were absorbed to MHC II molecules in the vaccinated mice (25). Importantly, bivalency of the DNA-encoded fu-
sion protein increased immune responses over the monovalent form (26).

Given the previous encouraging results in mouse tumor models, we wanted to investigate whether the principle could be extended to the infectious setting of influenza. HA is the primary target for Abs that neutralize influenza virus (28) and is also the most abundantly expressed protein on the surface of influenza virions. To maintain conformational determinants necessary for generation of strong Ab responses, extracellular portions of HA were inserted into vaccine-encoding plasmids (25). We demonstrate in this study that a single injection of mice with the DNA vaccine induced a complete Ab-mediated protection against virus within 8 d. Furthermore, the vaccine induced T cells capable of mediating cross protection against other strains of influenza. The vaccine could be produced and administered within a few weeks, as demonstrated in this study for a vaccine encoding HA from the Mexican flu pandemic of 2009.

Materials and Methods

Molecular cloning

Vaccine molecules were constructed by inserting targeting units and antigenic units into the cloning sites of the previously described plNOH2 CMV-based expression vector (25, 26). Targeting units were either a single-chain variable fragment (scFv) specific for MHC II molecules (H-2D) or an scFv specific for the haptens 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) (negative control) (25). As the antigenic unit, a fragment encoding aa 18–541 of HA from influenza A/PR/8/34 (H1N1) (PR8) was cloned using plasmid HAwt-pCMV (kind gift from Harald von Boehmer) as a template. The aa 18–541 of HA from influenza A/California/7/2009 (H1N1)–like strain [X-179A]; GlaxoSmithKline (1:100 in PBS), blocked with 0.1% BSA in PBS, and incubated overnight at 4°C with titrated amounts of sera from mice assayed individually (n = 6/group). Abs in sera were detected with biotinylated anti-IgG (A2429; Sigma-Aldrich), anti-IgG1 (553599; BD Pharmingen), anti-IgG2a (553502; BD Pharmingen), anti-IgG2b (553503; BD Pharmingen), anti-IgG2c (553515; BD Pharmingen), or anti-IgM (553419; BD Pharmingen), followed by streptavidin alkaline phosphatase and development as described above. Purified H36-4-52 mAb (IgG2a) was used as standard for quantification of IgG in the titration experiment (Fig. 2G). For other experiments, titers are given, defined as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five times SEM).

Detection of anti-HA Abs in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluids (BALF) were harvested from anesthetized (Hynpnom/Dormicum; 0.05 ml working solution/10 g s.c.) and exanguinated mice (n = 5/group) and added in triplicates to 96-well plates coated with PR8 as described above. Abs against PR8 were detected with alkaline phosphatase–conjugated anti-IgG Abs (A2429; Sigma-Aldrich). Plates were developed and read as described above.

Flow cytometry

MHC II 1E2–transfected L-cell fibroblasts (CA36.2.1 (E2), CA36.1.3 (E2), and CA25.8.2 (Dd)) (32) were Fc γ-R-blocked by incubation with 50% heat-aggregated rat serum and 0.1 mg/ml 2.4G2 mAb and then stained with affinity-purified vaccine proteins (10 μg/ml), biotinylated anti-HA Ab (H36-4-52; kind gift from Siegfried Weiss) (29) and streptavidin–HRP (554061; GE Healthcare). The staining solution also included PerCP–Cy5.5–conjugated anti-CD11b Abs (550993; BD Pharmingen), PE–conjugated CD11c Abs (553802; BD Pharmingen), FITC–conjugated anti-CD19 Abs (553785; BD Pharmingen), and Pacific Blue–conjugated CD4–57-0042-82 (eBioscience) and CD8–specific Abs (558106; BD Pharmingen) (CD4+, CD8+–constituted dump gate). Splenocytes were run on an LSRII flow cytometer (BD Biosciences) and data analyzed with the FlowJo software (version 7.6; Tree Star).

The peritoneal cavity of BALB/c mice was flushed with PBS, and single-cell suspensions prepared as above. Peritoneal cells and splenocytes (1 × 106 cells/well) were resuspended sequentially with vaccine proteins (10 μg/ml), biotinylated anti-human C3 Abs (B3773; Sigma-Aldrich), and streptavidin–allophycocyanin (554067; BD Pharmingen). The staining solution also included PerCP–Cy5.5–conjugated anti-CD11b Abs (550993; BD Pharmingen), PE–conjugated CD11c Abs (553802; BD Pharmingen), FITC–conjugated anti-CD19 Abs (553785; BD Pharmingen), and Pacific Blue–conjugated CD4–57-0042-82 (eBioscience) and CD8–specific Abs (558106; BD Pharmingen) (CD4+, CD8+–constituted dump gate). Splenocytes were run on an LSRII flow cytometer (BD Biosciences) and data analyzed with the FlowJo software (version 10.0.5; Tree Star).

Hemagglutination-inhibition assay

Equal volumes of sera from individual mice (n = 6/group) were pooled and treated with receptor-destroying enzyme (II) (Denka Seiken) at 37°C for 20 h. The enzyme was deactivated by incubation at 56°C for 40 min. Sera

ELISA for detection of serum anti-HA Abs

Blood samples were collected from mice by puncture of the saphenous vein and sera isolated by two successive centrifugations for 5 min at 13,000 rpm. The 96-well plates were coated with inactivated PR8 virus (Charles River Laboratories) (1:1600 in PBS) or Pandemrix (Ag suspension with A/California/7/2009 [H1N1]–like strain [X-179A]; GlaxoSmithKline (1:100 in PBS), blocked with 0.1% BSA in PBS, and incubated overnight at 4°C with titrated amounts of sera from mice assayed individually (n = 6/group). Abs in sera were detected with biotinylated anti-IgG (A2429; Sigma-Aldrich), anti-IgG1 (553599; BD Pharmingen), anti-IgG2a (553502; BD Pharmingen), anti-IgG2b (553503; BD Pharmingen), anti-IgG2c (553515; BD Pharmingen), or anti-IgM (553419; BD Pharmingen), followed by streptavidin alkaline phosphatase and development as described above. Purified H36-4-52 mAb (IgG2a) was used as standard for quantification of IgG in the titration experiment (Fig. 2G). For other experiments, titers are given, defined as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five times SEM).
were titrated and added in triplicates to 96-well plates. Diluted allantoic fluid containing 4 hemagglutinating units PR8 virus was added and plates incubated for 40 min at room temperature. Turkey RBCs (1%) were added to wells, and the plates were read for hemagglutination-inhibition (HI) 45 min later. HI was scored as the highest dilution of antisera giving a complete inhibition of hemagglutination. Validity of results was confirmed by the positive control serum (influenza PR-8 antisera; Charles River Laboratories) reaching its predicted titer and the negative control serum giving a titer of <$2^3$.

**Microneutralization assay**

Viral TCD50 was determined by the Reed-Muench method (33). Inclusion of TPCK trypsin did not significantly improve influenza infectivity and was therefore omitted in these assays. Equal serum volumes from individual mice (n = 6/group) were pooled and treated with receptor-degrading enzyme (II) as described above. Two-fold duplicate dilutions in virus diluent (DMEM supplemented with 1% bovine albumin fraction V, antibiotics, and 0.02 M HEPES) were set up in triplicates in 96-well plates. Four control wells of virus in diluent and diluent alone (cells only) were included on each plate. Fifty microtiter 100 × TCD50 virus was added to each well, except the cells only wells, and plates were incubated for 2 h at 37°C in a 5% CO2 humidified atmosphere. Madin-Darby canine kidney cells (2 × 10⁶) were added to each well, followed by incubation for 20 h at 37°C and 5% CO2. The monolayers were washed with PBS, fixed in cold 80% acetone for 10 min, and viral proteins detected by an ELISA using biotinylated mAb against the influenza nucleoprotein (HB65; American Type Culture Collection) and streptavidin-alkaline phosphatase. Plates were read as described above, with a Tecan reader using the Magellan v5.03 program.

**Viruses**

PR8 and A/California/07/09 (H1N1) (Cal07) were kindly provided by Dr. Anna Germundsson (The National Veterinary Institute, Oslo, Norway). The viruses were propagated by inoculating virus into the allantoic cavity of 10-day-old embryonated chicken eggs. Identity of the injected viruses was confirmed by sequencing of HA. Allantoic fluid was harvested and confirmed negative for bacterial contaminations. TCD50 was determined.

**Mice**

Six- to 8-week-old female BALB/c mice (Taconic Farms), 6–10-week-old B10.D2-H-2b (Harlan UK), and 6–8-week-old female BALB/C Nude (Taconic Farms) were used. Animals were housed under minimal disease conditions. All animal experiments were approved by the National Committee for Animal Experiments (Oslo, Norway).

**Vaccination and viral challenge**

Mice were anesthetized by s.c. injection of Hypnorm/Dormicum (0.05 ml working solution/10 g) and shaved in the lower back region. Twenty-five microliters plasmids (purified from the Endofree Qiagen kit [Qiagen]), dissolved in NaCl, was injected intradermally on each flank of the mouse, immediately followed by skin electroporation (EP) with DermaVax (Celllectis) (34, 35). A total of 25 μg DNA, divided into two equal doses, was injected per mouse, except in the dose titration experiments (n = 6/group).

Groups of anesthetized mice (n = 4/group) were injected intranasally with titrated doses of influenza virus in allantoic fluid (1, 10⁴, 10⁵, 10⁶, or 10⁷ TCD50) (n = 4/group) and LDI0 determined in accordance with the Reed and Muench method (33). For assessing the effect of vaccination, anesthetized mice were infected with 5x TCD50 PR8 (2.0 × 10⁶ TCID) or Cal07 (1.0 × 10⁶ TCID) in 10 μl/ nostril. Mice were monitored for weight loss (n = 6/group), with an end point of 20% weight reduction, as required by the National Committee for Animal Experiments. Mice reaching the >20% weight loss were euthanized by cervical dislocation. In the experiment in which mice were challenged 10 mo after vaccination (n = 6/group, except for NaCl, in which n = 5), a group of previous PR8 survivors was included as a positive control (n = 3).

**Quantitative PCR**

Noses of mice (n = 4) were flushed with 1 ml PBS/BSA (2%) and RNA extracted using NucliSens easyMag (BioMérieux). Quantitative RT-PCR was performed with samples in triplicates and with the following primers (Qiagen OneStep RT-PCR kit): 50°C (30 min), 95°C (2 min), followed by 45 cycles of 95°C (15 s) and 55°C (30 s) using a Stratagene Real-time Machine (Stratagene) with primers 5’-GAC CRA TCC TGT CAC CTC TGA C-3’ and 5’-AGG GCA TTY TGY AAG AAK CTA C-3’ and probe 5’-TGC AGT CCT CGC TCA CTG GGC ACG-3’ (MedProbe), all in accordance with the Centers for Disease Control and Prevention protocol (http://www.who.int/csr/resources/publications/swineflu/CDCrealTimeRTPCRprotocol_20090). H&E staining

Lungs were collected at day 7 because this was the time point at which most mice vaccinated with NaCl or anti-NIP-HA had to be euthanized for humane reasons. The excised lungs were fixed in formalin, embedded in paraffin, and stained with H&E. Tissue sections were examined as a Leica DMRB microscope (Leica Microsystems; original magnification ×10/0.75). A human pathologist experienced in evaluations of lung tissues from patients deceased from severe influenza was consulted for interpretation of images. Mice were scored as displaying lung pathology if the following signs were observed: histiocytic alveolitis, interstitial edema, and thickening of alveolar septum.

**ELISPOT assay**

Multiscreen HTS plates (Millipore) were coated with 12 μg/ml anti-mouse IFN-γ (AN18) (36) and blocked with complete tissue culture medium (RPMI 1640 containing 10% FCS and supplements). Single-cell suspensions (n = 6/group) were prepared by washing individual spleens through a cell strainer. The suspended cells were incubated 7 min on ice with ACT lysis buffer and washed three times with DMEM. The viable cells were added to multiscreen plates in 10⁴, 5 × 10⁴, and 2.5 × 10⁵ cells/well and stimulated with HNTNGVTAAACHEG, IYSTVASSL, or an irrelevant control peptide (GYKDGKEYN1) (0.8 μg/ml; Proimmune) before incubation with biotinylated anti-mouse IFN-γ (1 μg/ml; XM11.2; BD Pharmingen) and streptavidin–alkaline phosphatase (1:3000; GE Healthcare). IFN-γ-producing cells were detected by the BCIP/NBT kit (Zymed Laboratories), and an automated analysis of spots was performed using the Zeiss KS ELISPOT system v 4.3.56 (Carl Zeiss).

**Serum transfer**

Mice were vaccinated once with 25 μg DNA/EP (n = 6/group). Two (Fig. 5C) or 4 wk (Fig. 7A) later, mice were anesthetized by an s.c. injection of Hypnorn/Dormicum (0.05 ml working solution/10 g) and blood harvested by cardiac puncture. Sera were collected by two successive 5-min centrifugations at 13,000 rpm. Pooled sera (n = 6) were transferred into naïve BALB/c by i.v. injection (200 μl volume). Twenty-four hours later, mice were challenged with 5× LD₅₀ PR8 and monitored for weight loss (n = 8/group).

**T cell depletion**

Mice were vaccinated once with 25 μg DNA/EP (n = 6/group). From day 12, groups of mice vaccinated with anti–MHC II–HA were injected every other day i.p. with 400 μg either purified anti-CD4 (GK1.5; American Type Culture Collection) (37) or anti-CD8 (TIB105; American Type Culture Collection) (38), both, or control mAbs (SRFS-B6 and Y13-238). On day 14, mice were challenged with PR8 and monitored for weight loss. At the day of termination postchallenge, mice were euthanized by cervical dislocation and spleens harvested for assessment of in vivo T cell depletion. Single-cell suspensions were prepared and blocked as above, followed by staining with Abs against CD3 (PE-conjugated, 1505-09; Southern Biotechnology Associates), CD8 (allophycocyanin-conjugated, 1550-11; Southern Biotechnology Associates), CD4 (FITC-conjugated, 1540-02; Southern Biotechnology Associates), or isotype-matched controls (PE-conjugated hamster IgG1 [553972; BD Biosciences], allophycocyanin-conjugated rat IgG2a [553932; BD Biosciences], FITC-conjugated rat IgG2a [11214C; BD Pharmingen]). The degree of depletion was calculated to be >85% for CD4⁺ T cells and ≈95% for CD8⁺ T cells, respectively. Representative results of stained splenocytes from mice depleted with Abs against CD4 or CD8 are shown in Supplemental Fig. 2A and 2B.

**T cell transfer**

Mice were vaccinated once with 25 μg DNA/EP. Ten days later, mice were euthanized by cervical neck dislocation, and spleens and draining lymph nodes (LNs) were collected. Single-cell suspensions from spleen were prepared as described above, whereas LNs were directly washed through a cell strainer and washed three times in DMEM. T cells were negatively selected by using the Dynabeads Mouse Pan B (B220) kit (114.1D4: Invitrogen) and stained for FACS analyzes as described above with Abs against CD8α (Pacific Blue–conjugated, 558106; BD Biosciences), CD4 (allophycocyanin-conjugated, 1540-11; Southern Biotechnology Associates), CD45R/B220 (FITC-conjugated, 1665-02; Southern Biotechnology Associates), and CD3 (PE-conjugated, 1530-09C; Southern Biotechnology Associates). The purity
of the T cell solutions used for transfer was found to be 95–98% (see Supplementary Fig. 3C–E for representative FACS stainings). The purified T cells ($5 \times 10^6$/mouse) were injected i.v. into naive BALB/c mice ($n = 6$/group). Twenty-four hours later, mice were challenged with $5 \times LD_{50}$ of PR8 and monitored for weight loss.

**Statistical analyzes**

Statistical analyzes of Ab responses in sera were performed using one-way ANOVA and Bonferroni multiple comparison test. All other analyzes were performed using the nonparametric Mann–Whitney U test (GraphPad Software). For experiments with viral challenge, the statistical testing was performed on data from day 7. The $\alpha$ level was set to 0.05 for all analyzes.

**Results**

**Construction and characterization of the MHC II–targeted influenza vaccine**

The DNA-encoded vaccine proteins are homodimers, each chain consisting of: 1) a targeting unit; 2) a dimerization unit derived from the hinge and C$_{\gamma}^3$ exons of human IgG3; and 3) an antigenic unit (Fig. 1A). The antigenic unit, HA of PR8, was truncated at aa 18 and 541 to remove transmembrane sequences that could otherwise have caused problems with secretion. The shortened HA (aa 18–541) gene was fused via the hinge and C$_{\gamma}^3$ exons of human IgG3 domain (25) to the scFv of an mAb specific for a mouse MHC II molecule (I-E$^\beta$) expressed on B cells, macrophages, and dendritic cells. The dimeric vaccine is thus denoted anti–MHC II–HA. The construct was inserted into the pLNOH2 vector (39) and expressed under the control of a CMV promoter and an Ig-derived signal sequence. To measure the effect of MHC II targeting, we prepared a control vaccine identical to anti–MHC II–HA, but in which the MHC II–specific scFv was exchanged with an scFv specific for the synthetic hapten NIP (anti-NIP–HA). A standard vaccine containing the HA subunit (aa 18–541) alone served as an additional control (HA).

By EM, the monomeric HA subunits of the dimeric vaccine protein appeared to be independently arrayed without evidence of mutual interaction, in contrast to the trimeric association of membrane
HA on influenza virions. The vaccine proteins displayed an inherent flexibility, allowing for some variation of subunit orientation (Fig. 1B).

Cells transfected in vitro with either the anti–MHC II–HA or anti-NIP–HA constructs secreted dimeric vaccine proteins with expected sizes and properties (Fig. 1C, 1D). However, transfection of 293E cells with the plasmid encoding truncated HA alone yielded only low amounts of protein. In an attempt to increase the amount of secreted HA proteins, the Ig-derived signal peptide for this construct was replaced with the naturally occurring HA signal peptide. This modification resulted in increased secretion by transfected cells, comparable to that observed for the other constructs (Fig. 1D, right panel). The anti–MHC II targeting unit was demonstrated to specifically bind MHC II-transfected fibroblasts (Fig. 1E) and CD19+ B cells, CD11c+ dendritic cells, and CD11b+ macrophages from BALB/c spleens (Fig. 1F). As expected, anti-MHC–HA did not bind splenocytes from C57BL/6 mice that do not express I-E MHC II molecules (data not shown).

Previous reports have demonstrated that ligation of MHC II molecules results in signaling. However, the downstream effects appear to differ with cell type and developmental stage, as well as with specificity of the MHC II ligand (40–43). We therefore tested if anti–MHC II–HA added to ex vivo peritoneal cells or splenocytes could upregulate expression of costimulatory CD80 and CD86 on CD19+ B cells, CD11c+ dendritic cells, and CD11b+ macrophages from BALB/c spleens (Fig. 1F). As expected, anti-MHC–HA did not bind splenocytes from C57BL/6 mice that do not express I-E MHC II molecules (data not shown).

Targeting of HA to MHC II molecules enhances Ab responses BALB/c mice were injected once intradermally with the different DNA vaccines, immediately followed by EP of the injected site. EP increases the transfection efficiency of DNA (35, 44), resulting in increased vaccine production and local inflammation (45, 46). Vaccination with anti–MHC II–HA induced significantly higher amounts of Abs than did either anti-NIP–HA or HA, as detected in ELISAs against influenza virus (PR8). Increased Ab levels were observed on splenocytes (data not shown) or B cells from the peritoneal cavity (Supplemental Fig. 1). However, anti–MHC II–HA enhanced expression of CD80 and CD86 on CD11b+ and CD11c+ peritoneal cells, but the magnitude of upregulation differed for the two cell types (Supplemental Fig. 1). Similar results were obtained from two mice (Supplemental Fig. 1). Surprisingly, the nonspecific vaccine molecules (anti-NIP–HA) had a partial activating effect compared with medium alone, perhaps due to the HA or CH3 moieties of the molecule. However, anti–MHC II–HA was overall superior at inducing CD80 or CD86 expression compared with anti-NIP–HA. These results are generally consistent with previous reports demonstrating that ligation of MHC II molecules can signal APCs (40–43).

Targeting of HA to MHC II molecules induces complete protection against influenza Given the strong Ab responses elicited by DNA immunization with anti–MHC II–HA, we next tested whether such vaccination conferred protection against a viral challenge. Mice were vaccinated once and challenged either 14 d (Fig. 3A, 3B) or 10 mo (Fig. 3C) later with a lethal dose of PR8 virus. Mice that received saline or anti-NIP–HA rapidly lost weight and had to be euthanized by day 7 (Fig. 3A, 3C). In contrast, mice vaccinated with anti–MHC II–HA maintained their weight and showed no signs of disease. PCR analysis of viral loads in nasal washes showed that mice immunized with anti–MHC II–HA for the most part had cleared the virus infection by day 4 and completely so by day 6 (Fig. 3B). Lungs harvested from saline- or HA-vaccinated mice displayed histiocytic alveolitis and interstitial pneumonia. In contrast, mice vaccinated with anti–MHC II–HA had healthy lungs. Anti-NIP–HA–vaccinated mice also displayed lung pathology, but less severe than that of the NaCl and HA mice, consistent with partial clearance of virus in some mice (Fig. 3D, 3E). Anti–MHC II–HA DNA vaccination was also protective in B10.D2 mice, demonstrating that the observed immune responses were not strain specific (Fig. 3F). Vaccination in the absence of EP failed to induce Abs and protection (Fig. 3G, 3H).

Targeted DNA HA vaccines can be produced and employed within weeks During the spring of 2009, the reassortant H1N1 Mexican swine flu emerged as a pandemic threat. We used this occasion to test how rapidly an MHC II–targeted vaccine for this virus could be established. As soon as the HA sequence was available online, we inserted this sequence, Cal07, into the vaccine-encoding plasmids, replacing the PR8 HA. Within 3 wk, mice were vaccinated with the tailored Cal07 vaccine and monitored for Abs responses (Fig. 4A, 4B). MHC II targeting improved IgG1 and IgG2a Abs responses against Cal07. In a separate experiment, mice were challenged 14 d after a single vaccination with the virulent Cal07 virus (Fig. 4C). Again, MHC II targeting was essential for complete protection against disease. Thus, the targeted DNA vaccine strategy works for at least two different H1 strains of influenza virus (PR8 and Cal07), and tailored vaccines can be generated and tested in model systems within weeks of an influenza outbreak.

Mice are protected against influenza within 8 d after a single vaccination, and protection can be transferred with serum To determine how fast an MHC II–targeted immunization with HA could confer protection, mice were vaccinated with anti–MHC II–HA 14, 12, 10, 8, 6, 4, 2, or 0 d prior to a lethal challenge with PR8. Eight days after vaccination, mice had acquired protective immunity against viral challenge (Fig. 5A). Protection occurred at...
the same time as Abs rapidly increased in sera (Fig. 5B), indicating a correlation between protection and Ab responses.

To directly test if Abs conferred protection, sera from six mice immunized 14 d earlier with anti–MHC II–HA were transferred into naive mice that were then challenged with PR8 virus and monitored for development of disease. Transfer of 0.2 ml serum completely protected against a viral challenge (Fig. 5C).

**Targeting of HA to MHC II molecules enhances T cell responses that contribute to protection against viral challenge**

For assessment of T cell responses, splenocytes from BALB/c mice, immunized once with either the APC-targeted anti–MHC II–HA or nontargeted controls, were stimulated with a class II–restricted peptide [HNTNGVTAACSHEG (47)], a class I–restricted peptide [IYSTVASSL (48)], or a control peptide. Targeting to MHC II molecules on APCs enhanced the levels of HA-reactive IFN-γ–producing T cells (Fig. 6A, 6B).

For an assessment of T cell contributions to protection, mice vaccinated once with anti–MHC II–HA (PR8) were treated from day 12 with injections of depleting Abs against CD8, CD4, or both. The efficiencies of depletion were determined to reach 85 and 93% for CD4+ and CD8+ T cells, respectively (see Supplemental Fig. 2A, 2B for representative FACS stainings). The absence of weight loss in T cell–depleted mice challenged with virus on day 14 (Fig. 6C) was not surprising because Abs alone protected against influenza, as demonstrated by serum transfer (Fig. 5C). Thus, even though HA-specific T cells were required for the induction of anti-HA Abs (Fig. 2K), they were not needed for the elimination of virus. However, this experiment did not exclude a protective role of T cells because the protective anti-HA Abs present in this experimental setting could have disguised their importance. We therefore transferred B cell–depleted LN and spleen cells from immunized mice into naive recipients that were then challenged with virus. T cell recipients gradually lost some weight for the first 7 d, but then recovered, indicating that the anti–MHC II–HA-induced HA-specific T cells independently can confer protection against PR8 influenza virus (Fig. 6D, Supplemental Fig. 3A–E). Delayed protection is typical of T cell–mediated protection against influenza (49) because the initial entry of virus into host cells will not be prevented by T cells. In conclusion, a single anti–MHC II–HA DNA vaccination induces protective levels of both Abs and T cells (Figs. 5C, 6D).

**Targeted DNA HA vaccines induce T cells that cross-protect between H1 strains**

Anti-HA Abs generally do not cross-react between different strains of influenza virus. Consistent with this, sera from mice vaccinated with anti–MHC II–HA (PR8) failed to recognize Cal07 virus in a microneutralization assay (Supplemental Fig. 4), and transfer of sera from mice vaccinated with anti–MHC II–HA (Cal07) did not protect recipients against a challenge with PR8 (Fig. 7A). However, despite the lack of serological cross-reactivity, there is considerable sequence homology between the HAs of these two immunization with various doses of DNA (n = 6/group). (H) IgG anti-PR8 in BALF from mice at day 125 after a single immunization (n = 5/group). (I) BALB/c mice were vaccinated intradermally with 25 µg DNA in the absence of EP (n = 6/group). Blood samples were collected at various time points and IgG anti-PR8 assayed in ELISA. (J) B10.D2 H-2d mice were immunized once with 25 µg DNA/EP (HA from PR8) or NaCl. Sera were assayed for IgG anti-HA Abs. (K) Athymic BALB/c nude mice were DNA/EP-immunized once with 25 µg of the plasmids indicated above (n = 4/group). Serum samples were examined for IgG anti-HA in ELISA using PR8 as coat. No significant differences between groups were observed.

**FIGURE 2.** DNA vaccine format that targets HA protein to MHC II increases Ab responses. (A–F) BALB/c mice were vaccinated intradermally with 25 µg of the indicated plasmids (key at top) (n = 6/group), followed by EP, as indicated by arrows (↑). Values given are mean ± SEM. Serum levels of total IgG (A), IgG1 (B), and IgG2a (C) anti-PR8 Abs, as measured in ELISA. (D) Serum HI titers. (E) Serum samples from days 14 and 42 after a single immunization were assayed in a microneutralization assay (PR8 virus) (n = 6/group). Dotted lines indicate threshold for positive neutralization. (F) BALB/c mice were immunized three times (↑) and tested for anti-HA Abs in ELISA (n = 6/group). (G) IgG anti-PR8 following a single
H1 strains, and they share two out of three HA T cell epitopes described in BALB/c mice, a CD8 epitope (aa 533–541/Kd) and a CD4 epitope (aa 111–119/I-Ed) (PR8 numbering) (47, 48, 50). Therefore, because anti–MHC II–HA (PR8) vaccination induced T cell–mediated protection against homologous PR8, we asked whether MHC II–targeted vaccination could induce T cell–mediated cross-protection between the two different H1 viruses. Mice were vaccinated with anti–MHC II–HA (PR8) and then challenged with the Cal07 virus. Mice initially showed moderate weight loss, followed by a regain of weight (Fig. 7B). Conversely, mice vaccinated with anti–MHC II–HA (Cal07) and challenged with PR8 lost some weight but then recovered (Fig. 7C). In both cases, the protection was dependent on MHC II targeting. Because Abs did not cross-protect, it is highly likely that T cells confer vaccine-elicited cross-protection between the two H1 strains. Immunization with HA alone was not included in all of the experiments with viral heterochallenge because such immunization did not confer protection even against homologous challenge (Figs. 3D–F, 4C).

**FIGURE 3.** MHC II–targeted HA vaccine delivered as DNA provide short- and long-term protection against a lethal challenge with influenza virus. (A and B) BALB/c mice were immunized once with 25 µg plasmids/EP as indicated (key at top) (n = 6/group), challenged 14 d later with a lethal dose of influenza virus (PR8), and monitored for weight loss (A) and virus (B) in nasal washes at days 4 and 6 after challenge (quantitative RT-PCR) (n = 4/group). (C) Mice were immunized once 10 mo earlier with the indicated plasmids (25 µg/EP) (n = 6/group), challenged with PR8, and monitored for weight loss. Mice surviving a previous challenge with PR8 influenza virus was included as positive control (n = 3) (○). (D and E) Representative HE-stained sections of lungs (original magnification ×10) collected 7 d postchallenge. Mice (n = 6/group) were vaccinated once as indicated (25 µg plasmids/EP) and challenged 14 d (D) or 10 mo (E) later with PR8. Frequency of lung pathology (histiocytic alveolitis, interstitial edema, and thickening of alveolar septum) is graphically summarized in the right panels. p < 0.05 for anti–MHC II versus anti-NIP–HA, HA, and NaCl. (F) B10.D2 H-2d mice were immunized once with 25 µg DNA/EP (HA from PR8) or NaCl. Three weeks after vaccination, mice were challenged with PR8 virus and monitored for weight loss (n = 5/group). BALB/c mice vaccinated in the absence of EP were challenged 14 d (G) or 10 mo (H) later with a lethal dose of influenza PR8 and monitored for weight loss. Data in (G) and (H) are not significant. Ct, Threshold cycle.

**FIGURE 4.** Extension to Cal07. (A–C) BALB/c mice were DNA/EP vaccinated with the indicated plasmids encoding HA from Cal07 (n = 6/group). Serum levels of IgG1 (A) and IgG2a (B) anti-HA Abs measured in a Cal07 ELISA. Values given are mean ± SEM. (C) BALB/c mice were DNA/EP vaccinated once with 25 µg of the indicated plasmids encoding HA from Cal07 (n = 6/group), challenged 14 d later by a lethal dose of Cal07, and monitored for weight loss.
monitored.

Twenty-four hours later, mice were challenged with PR8 virus and the weight (42, 43). 4) APCs primed with anti–MHC II–HA vaccine cross-linking of MHC II molecules induce APC maturation (42, 43). 3) Such binding can result in increased expression of costimulatory CD80/CD86 proteins bind MHC II molecules on APC. This choice was based upon the previous findings shown. (B) Immediately prior to challenge, serum samples were obtained, and anti-HA IgG was measured in a PR8 ELISA. (C) Protection by serum transfer. Total of 200 µl serum from BALB/c mice vaccinated with anti–MHC II–HA (PR8)/EP (gray upward-facing triangles), HA (PR8)/EP (gray downward-facing triangles), and NaCl/EP (gray squares) were only immunized at day 14 (n = 6/group). (A) Weight following viral challenge, data for day 7 are shown. (B) Mice were immunized with 25 µg anti–MHC II–HA (PR8)/EP (gray circles) or NaCl/EP (gray squares) 14 d earlier (n = 6/group) were injected i.p. into naive BALB/c mice (n = 8/group). Twenty-four hours later, mice were challenged with PR8 virus and the weight monitored.

Discussion

In response to pandemic influenza, it is important to quickly produce large quantities of vaccines that rapidly can induce protective Abs. We describe in this study a DNA vaccine against influenza, in which transfected cells serve as production factories for secreted dimeric molecules that target HA to MHC II molecules on mouse APC. A single immunization induced within 8 d large amounts of strain-specific Abs that protected mice against challenges with the homologous H1 virus. Ab production and protection was long-lasting. In addition, the vaccine enhanced the induction of T cells that cross-protected between different H1 strains of influenza.

Several factors could contribute to the accelerated and heightened immunogenicity of the MHC II–targeted HA DNA vaccine, given intradermally in combination with EP. Previous studies with a similar DNA vaccine (25), together with the present data, suggest the following mechanism: 1) EP of the injection site increases a similar DNA vaccine (25), together with the present data, suggests. 4) APCs primed with anti–MHC II–HA vaccine proteins are found in draining LNs of immunized mice (25). 5) Targeting of vaccine proteins to APCs results in a more efficient stimulation of CD4+ Th cells in vivo and in vitro, probably due to increased peptide loading of MHC II molecules (25). 6) The dimeric vaccine proteins expressing two antigenic HA units may more extensively cross link BCRs, resulting in more efficient B cell stimulation.

The specificity of anti-HA Abs obtained by the MHC II–targeted DNA vaccine resembled that observed after conventional egg-based vaccines in that Abs were strain specific. Interestingly, the enhancing effect of MHC II targeting was especially pronounced in enhancing effect of MHC II targeting was especially pronounced in inhibiting HI and microneutralization assays, indicating that the induced Abs preferentially were directed against the head of the HA molecule. As a mechanism for such a skewing of Ab specificity for HA, anti–MHC II–HA proteins could bridge a synaptic cleft (51) between MHC II+ APC and HA-specific B cells, resulting in enhanced B cell stimulation. Regardless of mechanism, HA-specific B cells needed help from T cells for production of anti-HA Abs.

The current vaccine molecules were targeted to MHC II molecules on APCs. This choice was based upon the previous findings by others that anti–MHC II Abs conjugated to Ags induce strong
Ab and T cell responses against the Ag (12, 17, 19, 52). There may indeed exist even better targets on APC than MHC II for induction of strong Ab responses, and further studies in this respect are clearly warranted.

An important aspect of the present vaccine design is the inclusion of large protein Ags, such as HA, with an intact conformation. This is likely to be important for two reasons: 1) elicitation of B cell (Ab) responses against conformationally dependent antigenic determinants require large Ags; and 2) long polypeptides have an increased chance of generating peptides that fit any of the polymorphic MHC binding sites found within a species, facilitating the generation of T cell responses in any individual. In this respect, the described vaccine molecules can accommodate a range of large viral and bacterial protein Ags, the hitherto largest being the truncated HA (541 aa) used in this study.

Trimerization of HA has been described a prerequisite for transport from ER to the Golgi complex (53, 54). Despite this, we demonstrate efficient secretion of monomeric HA inserted into the fusion vaccine. A monomeric structure of HA was supported by EM that revealed single, nonaggregated, dimerized vaccine proteins in which HA appeared monomeric and flexible in their orientation within the dimer. Secretion could be facilitated by the Ig-like structure of the vaccine molecule, with monomeric HA trailing the aminoterminal scFv-CH3. Despite the monomeric structure of HA in a dimeric vaccine protein, the targeted fusion vaccine rapidly induced large amounts of Abs that upon serum transfer protected naive mice against influenza. These results demonstrate that the monomeric HA in a dimeric context displays relevant antigenic determinants corresponding to those expressed by virus. However, the HA vaccine proteins failed to agglutinate erythrocytes, indicating that they lacked the degree of multimerization required for detectable binding sialic acid in the hemagglutination assay (55). Alternatively, monomeric HA expressed in the dimeric fusion protein could have lost their binding site for sialic acid.

Vaccination with the nontargeted anti-NIP–HA control resulted in increased anti-HA immune responses compared with that elicited by HA alone. The enhanced immunogenicity of anti-NIP–HA, although inferior to that of anti–MHC II–HA, may be attributed to bivalency of HA and thus an ability to cross link anti-HA BCR. In addition, efficient secretion and the xenogeneic dimerization unit could both have contributed. Further studies are needed to assess the immune-enhancing properties inherent to the dimeric vaccine backbone, in the absence of targeting.

In the present experiments, DNA/EP vaccination with HA alone induced immune responses that were only barely detectable above background. This may appear surprising because others have previously observed more pronounced Ab responses, and protection against viral challenge, following DNA vaccination with HA. The discrepancy might be explained by the current study employing only a single immunization, a low injection volume, and a low DNA dose. By contrast, previous studies by others describe protection first after several immunizations (6, 7, 10), larger volumes (7, 9, 10, 56, 57), and larger amounts of DNA (9, 56–58). Another factor that may explain the discrepancy is that we have used a truncated HA in which most of the transmembrane and intracellular parts have been deleted. Others (6, 9, 10, 56–58) have used complete HA or selected influenza epitopes (7) for immunizations. Thus, the discrepancy might be explained by secreted HA (presumably monomeric) being less immunogenic than HA in a membrane-bound (presumably trimeric) form. Finally, the in-house pLNOH2 vector used in the present studies has not been optimized for high Ag expression in transfected cells, and it might well be that we would have observed a response to (truncated) HA with an improved vector.

An MHC II–targeted DNA vaccine for a novel influenza strain (Cal07) was generated and tested within 3 wk after the HA sequences were available online. Moreover, large amounts of DNA plasmids can be rapidly produced, compared with the slow and resource-demanding production of conventional egg-based influenza vaccines. Thus, in the face of pandemic threats, the technology described in this study should allow for rapid generation of large amounts of vaccine that induce protection in vaccinees within days. For human application, concerns may be raised with regard to the safety of DNA vaccination. Although DNA vaccines are not yet approved for prophylactic vaccination of humans, they have been licensed for rhadovirus in rainbow trout (59) and for the West Nile virus in horses (60). Therapeutic DNA vaccines delivered with EP have been experimentally tested in cancer patients without serious side effects (61), and several clinical trials with therapeutic DNA vaccines for cancer are currently ongoing in humans (62, 63).

It will be important to test if the triad of DNA injection, EP, and MHC II–targeted fusion protein vaccines can improve the efficacy

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**FIGURE 7.** Vaccine-induced T cells, but not Abs, cross-protect between PR8 and Cal07 H1 viruses. (A) Pooled sera from mice vaccinated as indicated 4 wk earlier (n = 6/group) were injected i.p. into naïve BALB/c mice (n = 8/group). Twenty-four hours later, recipients were challenged with PR8 and weight monitored. (B) Mice were vaccinated once with 25 μg of the indicated plasmids encoding HA from PR8 (n = 6/group), challenged with Cal07, and monitored for weight loss. (C) Mice were vaccinated once with 25 μg of the indicated plasmids encoding HA from Cal07 (n = 6/group), challenged with PR8, and monitored for weight loss.
of DNA vaccination in larger animals and humans. In that respect, the DNA dose-sparing effect observed with MHC II-targeted HA vaccines in mice is encouraging.

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Disclosures

G.G., A.B.F., and B.B. are inventors on patent applications filed on the vaccine molecules according to institutional rules through the Technology Transfer Offices of the University of Oslo and Oslo University Hospital. A.B.F. is part-time Chief Scientific Officer of Vaccibody. B.B. is head of the Scientific Panel of Vaccibody. The other authors have no financial conflicts of interest.

References


Supplemental Figure Legends:

**Figure S1. Up-regulation of CD80 and CD86 after co-incubation with vaccine proteins.** Cell suspensions from two BALB/c mice were prepared from peritoneal fluid, and incubated with 10μg/ml of the indicated vaccine proteins or medium. After 48h, the expression of CD80 and CD86 were examined in gated CD11b+, CD11c+ and CD19+ cells.

**Figure S2. Depletion efficiency after viral challenge.** Mice were immunized once with DNA/EP, and treated every other day from day 12 with depleting antibodies against CD4 (a) or CD8 (b). Stained splenocytes that were harvested at the termination of the experiment (day 7 after viral challenge) to assess the degree of depletion are shown. Black lines depict splenocytes from CD4 (a) or CD8 (b) depleted mice, whereas grey lines depict mice inoculated with isotype matched non-relevant antibodies. The shaded area depicts splenocytes from depleted mice that were stained with isotype matched control antibodies. The samples are representative within groups.

**Figure S3. MHC class II targeted DNA/EP immunization induces T cells capable of protecting against a lethal dose of influenza.** BALB/c mice were immunized with 25μg αMHCII-HA(PR8)/EP or NaCl/EP. Spleens and lymph nodes were harvested 10 days later. T cells from either source were negatively selected by magnetic beads and transferred i.v. into naive BALB/c mice (n=6/group), that were challenged 24 hours later with a lethal dose of PR8. For the NaCl-group, mice received pooled lymph node and spleen cells. (a) Weight at day 7 after challenge. *p=0.026 (NaCl vs. αMHCII-HA, LN), and **p=0.015 (NaCl vs. αMHCII-HA, spleen). (b) Survival curve after T cell transfer and challenge. Mice suffering a weight loss >20% were euthanized and scored as diseased. (c-e) FACS staining of the harvested cell population from LN prior to B cell removal (c) and after (d,e). (c) Pooled LN
cells from mice vaccinated with αMHCII-HA. (d,e) Pooled LN cells from mice vaccinated with αMHCII-HA (d) or NaCl (e) after the removal of CD45R+ cells from suspension by negative selection.

**Figure S4. Immunization induces neutralizing antibodies that do not cross-react with heterologous virus.** Mice were DNA/EP-vaccinated once with the indicated vaccines encoding HA from PR8, or NaCl (n=6/group). Serum samples from day 14 were assayed in a microneutralization assay with the heterologous Cal07 virus. No significant neutralization activity was detected. Neutralization of homologous virus is shown in Fig. 2e in paper version.