Background

In the latter half of the 1980s, it was demonstrated that directing protein antigens to surface molecules on antigen-presenting cells (APCs) significantly enhances immune responses following immunization (4-6). This foundational observation has been validated by subsequent studies (6-15). Initially, these experiments involved chemically linking antigens to antibodies that target specific APCs (4,6,16). More recently, genetic fusion methods have been preferred, combining an APC-specific targeting unit with the antigen (7,9,10). Various formats have been reported, including Ig-based constructs (7,9,10), single-chain Fv (scFv) formats (17,18), and chemokines (8,19,20) as APC-specific targeting units.

The type of immune response induced by targeted vaccines is influenced by the specific surface molecules they bind to on APCs (21). This is crucial, as the success of many vaccines relies on the induction of protective antibodies. To date, MHCII molecules appear to be the most effective APC targets for inducing antibody responses (5,7,13,17,22,23).

New vaccines are urgently needed to combat various complex pathogens of modern times, such as influenza, HIV, flaviviruses, tuberculosis, and malaria, where antibodies are a key component of protective immunity. Thus, targeting antigens to MHCII molecules could enhance the effectiveness of subunit vaccines. This strategy could also reduce the amount of vaccine required to induce protective immunity and decrease the number of doses needed. Additionally, the rapid induction of antibodies after a single MHCII-targeted vaccination could provide a crucial first line of defense against emerging pandemic viruses.

Targeting antigens to MHCII molecules may also be valuable in therapeutic contexts, where enhancing the production of antibodies with specific characteristics could be beneficial.

Experimental Details

Experimental Detail

Example 1

Human embryonic kidney (HEK) 293E cells and NSO cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, US). T18 is a human HLA-DR4 (DRAl, DRBl *0401)-restricted T cell clone specific for aa 40-48 of mouse Ig CK(25). The murine hybridoma

producing the anti-HLAII mAb (HKB1, IgM)(26) was a kind gift from Dr. Steinar Funderud, Oslo University Hospital. The anti-HLA-DR (L243, IgG2a)(27) was purchased from ATCC. The use of human peripheral blood mononuclear cells (PBMC) was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REC, 2014/1505). The cells were purified from whole blood by LymphoPrep density gradient centrifugation (Ny corned, Oslo, Norway). Construction of vaccine molecules

mRNA was isolated from the hybridomas HKB1 and L243 by use of Dynabeads® mRNA DIRECT TM kit (Dynal, Oslo, Norway), and cDNA synthesized using cDNA Synthesis Kit (Amersham Biosciences, Oslo, Norway). The V(D)J of HKB1 and L243 heavy and light chain genes were PCR amplified from the cDNA with degenerate primers complimentary to the leader sequence of VL and VH, and IgM, IgGl and CK. The PCR products for the light chain and heavy chain were ligated into individual pGEM®-T Easy vectors (Promega, Madison, WI, USA). To obtain scFv specific for HKB1 and L243, PCR reactions were run to reamplify the VH and VL domains from the pGEM®-T Easy vectors with specific primers including linkers and restriction enzyme sites (Bsml/BsiWI) for subcloning. 5'VH HKB1/L243: ggc gga ggt ggc tct ggc ggt ggc gga teg CAG ATC CAG TTG GTG CAG TCT and 3'VH HKB-1/L243: ga c gtacg a etc acc TGA GGA GAC TGT GAG AGT GG. Next, the various scFv were

Bsml/BsiWI digested and cloned into the Bsml/BsiWI cassette of pLNOH2(28) or pUMVC(29) (kind gift from Bob Weinberg, Addgene plasmid #8449) expression vectors to generate vaccine constructs with specificities for NIP, pan anti-HLAII, and anti-DR. The vaccine constructs were equipped with antigenic units by subcloning either the scFv-like homodimer CKCK, HA from influenza A/Puerto Rico/8/1934 (H1N1) (PR8), or HA from influenza A/California/07/2009 (H1N1) (Cal07)(23) on Sfil sites.

Western blot

Vaccine proteins containing the scFv CKCK as antigen, affinity purified (187.1 mAb column) from supernatants of stably transfected NSO cells, were run on a Novex 4-12% Tris- Glycine gel (Invitrogen, Life Technologies, CA, US) together with a Seegreen Plus2 Prestained Standard (LC5925, Invitrogen, Life Technologies, CA, US), blotted (Immun-Blot PVDF membrane, 162-0177, BioRad, CA, US) and incubated with biotinylated HP6017 (anti-human IgG CH3) (BioLegend, CA, US) and Streptavidin-HRP (RPN123 IV, GE Healthcare,

Buckinghamshire, UK). The membrane was developed with the ECL Western Blotting analysis system (RPN2109, GE Healthcare, Buckinghamshire, UK) and analyzed on a Kodak Image station 200R (LabX, Canada) with Molecular Imaging Software v 4.0.5.

One μg of DNA plasmids encoding vaccine constructs with HA as antigen were transiently transfected into 293E-cells (lxl05/well) by addition of Lipofectamin2000 (11668-019, Invitrogen, Life Technologies, CA, US). Supernatants were collected after 48 hours, and Western blot performed as described above. Proteins were detected with biotinylated H36-4-52 anti-HA mAb (kind gift from Siegfried Weiss,

Braunschweig)(30), and developed as described above.

Sandwich ELISA

ELISA plates (Costar 3590, Sigma- Aldrich, MO, US) were coated with either ^g/ml of mAb 187.1 (anti-mouse CK)(31), MCA878 (anti-human CH3) (AbD Serotec, CA, US), or NIP- BSA, blocked, and incubated with supernatants from transiently transfected 293E-cells (as above). Next, plates were incubated (^g/ml) with either biotinylated 187.1, or HP6017 (BioLegend, CA, US), or H36-4-52, and Streptavidin alkaline phosphatase (1 :5000) (GE Healthcare, Buckinghamshire, UK). Plates were developed using Phosphatase substrate (P4744- 10G, Sigma Aldrich, MO, US) dissolved in substrate buffer, and read with a Tecan reader (Tecan, Switzerland) using the Magellan v5.03 program.

Flow cytometry

Vaccine protein-staining ofPBMC: Freshly isolated human PBMC were stained sequentially with purified vaccine proteins, biotinylated HP6017 and streptavidin- APC (BD Pharmingen, CA, US). Cells were run on a BD FACScalibur system (BD Biosciences, NJ, US) and analyzed by FlowJo software (Version 7.6) (FlowJo, OR, US).

Vaccine protein-staining of cells from DQ2 transgene mice: Splenocytes from 3 DQ2 transgenic mice(32) were FcyR-blocked by incubation with 30% heat aggregated rat serum and 0,lmg/ml 2.4G2 mAb (Fisher Scientific, MA, US), and stained sequentially with vaccine proteins (lO g ml), biotinylated HP6017 (1 :2000) (042M4810, Sigma, MO, US) and Strep-PE ^g/ml) (554061, BD Pharmingen, CA, US). The staining solution also contained either FITC- conjugated mAb

against CD19 (4 g ml) (35-0193,9500, TONBO, CA, US), PerCP-Cy5.5 conjugated mAb against CDl lb ^g/ml) (66-0112-U100, TONBO, CA, US), or APC- conjugated mAb against CDl lc (20-0114-U100, TONBO, CA, US). Samples were run on LSRII (Becton Dickinson, NJ, US), and data analyzed as above.

Vaccine protein-staining of cells from larger animals: Blood collected from horses, cows, sheep, ferrets and pigs (n=3 for all species) on EDTA were added to Lympholyte (CL5120, Cedarlane Labs, Canada) (1: 1), and centrifuged for 40 min at 1500rpm. The lymphocyte layer was collected, washed with PBS, and plated out in 96-well plates (1x106 cells/well). Cells were blocked as above, and stained sequentially with vaccine proteins (10μg/ml), biotinylated mAb against HA (H36-4-52), and APC-conjugated Streptavidin (554067, BD Biosciences, NJ, US). Samples were run on LSRII, and data analyzed with the FlowJo software (Version 10.0.5). For staining of cells from ferrets, PE-conjugated mAbs against CD1 lb (clone Ml/70) (553311, BD Pharmingen, CA, US) was also included in the staining solution. For staining of cells from pigs, the vaccine proteins were detected with PE- conjugated mAbs against human IgG (2043-09, SouthernBiotech, AL, US). FITC -conjugated mAb against CD3s (559582, BD Biosciences, NJ, US), and mAb against CD11R3 (MCA2309, AbD serotec, CA, US) [detected with APC-conjugated mAb against IgGl (550874, BD

Pharmingen, CA, US)] were included in the staining.

T cell proliferation assays

APC were freshly isolated from PBMC (7 x 104/w) of HLA-DR4 (DRA1, DRB1 *0401) donors. Experiments were approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REC, 2014/1505). Irradiated APC (20 Gy) and mouse CK40-48- specific, DR4-restricted human CD4+ T cells (4 x 104/w)(25) were cultured in 96-well plates with titrated amounts of the various bivalent vaccines expressing CKCK. After 48 h, the cultures were pulsed for 16-24 h with 1 [3H]Thymidine, harvested, and [3H]Thymidine incorporated into DNA of proliferating cells was measured using a TopCount NXT Scintillation counter (Packard, Meriden, CT, US).

Specificity of vaccine proteins and HKB1 mAb for HLA class II

One μg of vaccine proteins expressing CKCK (purified on column with 187.1 mAb linked to Sepharose) (GE Healthcare, Buckinghamshire, UK) were mixed with HLAII-coated xMAP microbeads (LS2A01, One Lambda, CA, US) in 96-well plates, and incubated for 30 min at 22oC. Next, plates were washed three times, and samples incubated with PE-conjugated antibodies directed against the CH3-domain dimerization unit (409304, BioLegend, CA, US) for 30 min at 22oC. Samples containing HKBl were detected with PE-conjugated anti-mouse IgM (553517, BD Pharmingen, CA, US). Plates were washed twice, and samples resuspended in PBS before being read on a Luminex 100 flow analyzer (Luminex, TX, US) and analyzed with the HLA-Visual software (One Lambda, CA, US). The obtained HLA reactivity profile was then used to evaluate shared regions of the otherwise polymorphic HLA β-chains. Briefly, the complete HLA directory was downloaded (http://www.ebi.ac.uk/ipd7imgt/hla/), aligned with ClustalX2.1(33), and manually edited and annotated in GenDoc(34) to identify the likely HKB epitope. Representative PDB entries were contoured by APBS(35), and visualized using PyMOL Molecular Graphics System (Schrodinger, LLC). Animals

Six to eight week-old BALB/c and DQ2 transgene mice(32) (on a BALB/c background) were used. Mice were housed under minimal disease conditions at Oslo University Hospital, Oslo, Norway. Six- to eight week old pigs (Noroc) of both sexes were used (Noroc: 50% Norwegian landgris, 25% Norwegian Yorkshire and 25% Duroc). Weights at the start of the experiments ranged from 16-30kg, whereas weights at the termination of experiments ranged from 34-48kg. Pigs were housed at the Animal Production Experimental Centre, Norwegian University of Life Science, As, Norway. Experiments in mice and pigs were approved by the National Committee for Animal Experiments (Oslo, Norway). Ferret yearlings (female and male) were used, with weights ranging from 2,3 -7kg. Ferrets were housed at the Laboratory

Animal Facility, University of Copenhagen, Denmark. Experiments in ferrets were approved by the Danish Society for the Protection of Laboratory Animals (Copenhagen, Denmark).

Virus

Influenza A/Puerto Rico/8/1934 (H1N1) (PR8) was kindly provided by Dr. Anna Germundsson, The National Veterinary Institute, Norway. The virus was propagated by an inoculation into the allantoic cavity of 10-day-old embryonated chicken eggs. Allantoic fluid was harvested, confirmed negative for bacterial contaminations, and TCID50 determined.

Vaccination and viral challenge

Mice: Mice were anaesthetized by s.c. injection of Hypnorm/Dormicum (0,05ml working solution/1 Og), and vaccinated as previously described(23). Briefly, 25µ1

vaccine solution (0,5 mg/ml DNA) was injected intradermally (i.d.) on each flank of the mouse, immediately followed by skin electroporation (EP) with DermaVax (Cellectis, Paris, France). For viral challenge, LD50 was determined as previously described(23), and anaesthetized mice were intranasally infected with 5xLD50 of PR8 (2.0xl04TCID) in 20µ1 (IOµI per nostril). Mice were monitored for weight loss (n=6/group), with an endpoint of 20% weight reduction, as required by the National Committee for Animal Experiments.

Ferrets: Ferrets were immunized i.d. with 100µg DNA (pLNOH2-vector) in total, delivered by two separate injections of IOOµI (0,5mg/ml DNA) at each side of the lower back region.

Injection was immediately followed by skin electroporation (DermaVax, Cellectis, Paris, France).

Pigs: Pigs were DNA (pUMVC-vector) immunized either by i.d. needle injection followed by skin electroporation (DermaVax, Cellectis, Paris, France), or by i.d. jet delivery (Tropis, PharmaJet, DE, US). Serum ELISA

Sera were isolated from blood by two successive centrifugations for 5'at 13 000 rpm. 96- well plates were coated with inactivated PR8 (Charles River, MA, US) (1 : 1600 in PBS) or Pandemrix (antigen suspension with A/California/7/2009 (HlNl)v-like strain (X-179A), Glaxo Smith Kline) (1 : 100 in PBS), blocked with 0,1% BSA in PBS, and incubated overnight at 4oC with titrated amounts of sera. Antibodies in mouse sera were detected with biotinylated anti-IgG (A2429, Sigma Aldrich, MO, US), anti-IgGla (553599, BD Pharmingen, CA, US), or anti-IgG2aa (553502, BD Pharmingen, CA, US), followed by Streptavidin alkaline phosphatase (GE Healthcare, Buckinghamshire, UK) and development with Phosphatase substrate (P4744-10G, Sigma Aldrich, MO, US) dissolved in substrate buffer. The plates were read as above. Titers are given, defined as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five times SEM). In ferrets and pigs, HA-specific IgG antibodies in sera were detected with alkaline phosphatase conjugated anti-ferret IgG (LS-

C61240, LSBio, WA, US) and biotinylated anti-pig IgG (abl 12747, Abeam, Cambridge, UK), respectively. Plates were developed and analysed as above.

Microneutralization assay for influenza virus-specific antibodies

The microneutralization assay was performed as previously described(23). Briefly, sera were treated with RDE (II) (Denka Seiken, Tokyo, Japan), and twofold duplicate dilutions set up in triplicates. Fifty μΐ of 100xTCID50 virus (PR8 or Cal07) was added to each well, and plates incubated for 2h at 370C in a 5% C02 humidified atmosphere. MDCK cells (2x105) were added to each well, and plates incubated for 20 hours at 370C and 5% C02. 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 (HB65, ATCC, VA, US) and Streptavidin-alkaline phosphatase (GE Healthcare, Buckinghamshire, UK). Plates were read as described above.

Statistical analyses

Statistical analyses were performed using one way Anova and Bonferroni's multiple comparison test with the Graphpad Prism software (GraphPad Software Inc. version 5).

Results:

Cloning of scFv targeting units with specificity for human HLAII molecules

We have previously described efficient immune responses in mice elicited by a DNA vaccine format that encodes bivalent homodimers with N-terminal scFv targeting units specific for mouse MHCII molecules, a human IgG3-derived dimerization unit (shortened hinge + CH3), and with C-terminal antigenic units(17,23) (Fig. la). In order to test the efficacy of the vaccine format in larger animals and humans, we have here developed novel scFv targeting units directed against human HLAII molecules. The scFvs were derived from two different anti- HLAII specific mAb. The first mAb, L243 (mouse IgG2a), is specific for HLA-DR

molecules(27,36). The second mAb, HKB-1 (mouse IgM), reacts more generally with HLAII molecules and is denoted pan(p)HLAII-specific(26). The V regions of the two mAb were cloned from B cell hybridomas, sequenced, scFv were constructed and inserted into cassette vectors(17) that already contained the dimerization unit, and an antigenic unit [either two mouse CK

domains linked in a scFv-like format(37) or influenza HA(23)]. The resulting HLAII-targeted vaccines are designated OHLADR-CKCK, aHLADR-HA, apHLAII-CKCK and apHLAII-HA,

respectively. As non-targeted controls, we also prepared a vaccine where the HLAII-specific targeting unit was replaced with a scFv specific for the hapten NIP (αNIP-Oκ Oκ/αNIP-HA)(17,23). In addition, a plasmid expressing truncated HA antigens alone was used (the cytosolic tail of HA and part of the transmembrane region were removed to prevent retention in the cell membrane of transfected cells)(23).

Vaccine proteins secreted by cells transiently transfected with the vaccine encoding plasmids had expected sizes and reactivity with mAbs, as evaluated by ELISAs (Fig.

lb) and Western blotting (Fig. lc). Further, HLAII-targeted vaccine proteins bound all CD 19+ cells, about 37-47% of CDl lc+, and 5-6% of CDl lb+ cells among human PBMC (Fig. Id). The non-targeted control, (XNIP-CKCK, failed to significantly bind any of the cell populations, although some unspecific binding was observed.

Specificity of vaccine proteins for human HLAII molecules

The human leukocyte antigen class II (HLAII) loci are among the most polymorphic genes described in the human genome, with some HLAII genes having hundreds of identified alleles(38). We therefore assessed the novel vaccine proteins for their ability to bind 91 different HLAII molecules displayed on the surface of xMAP microbeads. apHLAII-CKCK bound 57/91 of the HLAII molecules tested (Fig. 2a and 13), including all but two HLA-DR molecules (33/35). Binding to HLA-DQ (12/29) and -DP (12/27) alleic variants was less frequent(39). As expected, (XHLADR-CKCK bound all HLA-DR molecules tested (35/35), but none of the HLA-DQ and -DP molecules. The negative control, (XNIP-CKCK failed to bind any HLAII molecules at all. The binding profile of apHLAII-CKCK was compared to IgM mAb HKBl, from which the scFv in the targeting units of apHLAII-CKCK were derived(26). The binding profiles of the HKB1 IgM and the vaccine proteins were overlapping, except that the original antibody weakly bound one more HLA-DQ molecule than did the vaccine protein (Fig. 13).

Given the ability of apHLAII-CKCK to bind members of all three series of HLAII molecules, a hitherto unappreciated shared structural feature between DP, DR and DP may comprise the binding epitope. Most of the genetic variation in HLAII molecules is located to the more distal

parts of the ectodomains, thereby affecting the unique peptide binding properties of these scaffold molecules. An exception is the monomorphic a-chain of HLA-DR, with only one functional allele(40). The inability to bind all HLA-DR molecules made us exclude the monomorphic a-chain from the analysis, and rather focus on the β-chain. Here, a global alignment of the IMGT/HLA database entries (PMID: 25414341) readily singled out a small region in the DR βΐ -domain that was unique for the two non-reactive DRBl* 11 :01

DRB 1 * 11 : 04 alleles. The key feature of this region is an Ala to Glu substitution in the otherwise conserved position of the β-chain. This feature was also shared with the non-reactive DP1 *02:01 chain, alluding to the incompatibility with a Glu in position 58 to allow for HKB1 binding (Fig. 2b). Moreover, a complex epitope architecture is likely as Glu58 is not shared with the non- reactive HLA-DQ8 (DQB1 *03:02), or the murine I-Ed (H-2EB*01 :01) that rather differ in neighbouring residues (Fig. 2c).

Vaccine proteins that target antigen to HLAII enhance proliferation of human T cells

The two CK domains paired in a scFv-like format in the antigenic unit contain an immunogenic T cell epitope (aa40-48) for which we have previously established a DR4- restricted (DRAl /DRB 1*0401) human CD4+ T cell clone(37,41). We could therefore test in vitro whether the different vaccine constructs could efficiently stimulate CK-specific human CD4+ T cells(25). Irradiated PBMC from DR4+ donors were pulsed with titrated amounts of vaccine proteins, and CD4+ T cell proliferation assessed. Strikingly, OHLAII-CKCK and

(XHLADR-CKCK were 1,000-10,000 fold more efficient at stimulating the CK-specific T cells as compared to (XNIP-CKCK (Fig. 3).

Targeted DNA vaccine induce protection against challenge with influenza virus in DQ2-transgene mice

Vaccine plasmids encoding HA and targeting units against pHLAII and HLA-DR (apHLAII-HA and aHLADR-HA), or non-targeted controls (aNIP-HA and HA alone), were transiently transfected into 293E cells. The secreted vaccine proteins displayed the expected sizes and reactivity with mAbs, as evaluated by ELISAs (Fig. 4a) and Western blotting (Fig. 4b). In accordance with results from the microbead assay (13), apHLAII-targeted vaccine proteins bound cells from DQ2 transgenic mice (DQA1 *05:01/DQB 1 *02:01). Consistent with results

from human PBMC, the vaccine proteins bound most CD 19+ cells and a substantial fraction of CDl l c+ splenocytes from DQ2-transgene mice, while aHLADR-HA and aNIP-HA failed to bind (Fig. 4c).

Next, DQ2 transgenic mice were DNA-vaccinated once intradermally (i.d.), immediately followed by skin electroporation to enhance cellular plasmid uptake. A single vaccination with apHLAII-HA significantly increased the levels of anti HA IgG in sera, compared to the non-targeted controls (aHLADR-HA, aNIP-HA, HA) (Fig. 4d). The strong increase in antibody levels was observed already 14 days after a single DNA immunization, and was particularly pronounced for IgGl (Fig. 4e), although IgG2a was also clearly increased (Fig. 4f).

At day 22 after vaccination, the mice were intra-nasally inoculated with a lethal dose of influenza virus (PR8). Weight monitoring demonstrated that DQ2-transgenic mice vaccinated with apHLAII-HA were protected against influenza (Fig. 4g). In contrast,

DQ2-transgeic mice vaccinated with aNIP-HA or HA alone succumbed to the infection.

Protection required expression of DQ2 since nontransgenic BALB/c mice vaccinated with apHLAII-HA were not protected against an influenza virus challenge. These results demonstrate that the pan HLA-specific HA DNA flu vaccine elicited a protection against viral infection that was contingent upon targeting to HLAII molecules.

aHLAII-targeted vaccines bind MHCII molecules expressed in several larger mammals Since the apHLAII- and aHLADR-targeted vaccine proteins bound a number of different human HLAII molecules, we tested if the vaccine proteins could cross-react with MHCII molecules of larger animals. The apHLAII-HA bound PBMC from horses, cows, sheep, ferrets and pigs (Fig.5a-b and 14), whereas aHLADR-HA bound the same species except cows (Fig. 14). The vaccine proteins failed to bind rabbit PBMCs and mouse splenocytes (not shown). A caveat is that a limited number of animals of each species were tested (n=3/specie), therefore, given the polymorphic nature of MHCII molecules, no conclusion about frequencies of binding can be made. In any event, the vaccine proteins are likely to bind a region of the of MHCII molecules that appears conserved between several larger mammals.

Strong antibody responses induced after MHCII-targeted DNA vaccination of ferrets and pigs

Given the staining results, ferrets were DNA-immunized once with l OC^g of plasmids encoding apHLAII-HA or non-targeted controls (aNIP-HA and HA) (Fig. 5c). The DNA was injected i.d., immediately followed by skin electroporation to increase DNA uptake. Ferrets immunized with apHLAII-HA had serum IgG levels that were significantly increased above that of the control groups (Fig.5c). The elevated antibody levels were evident from day 9, increased until day 29, and then gradually declined.

The weight of a ferret typically is 2-7 kg. In order to assess the vaccine efficacy in animals approaching human sizes, vaccinations were performed in Norwegian farm pigs.

Vaccines for DNA immunization of pigs were prepared in the pUMVC vector(29). Similar to ferrets, pigs were in a first experiment immunized with DNA by i.d. injection, immediately followed by skin electroporation. A single DNA immunization with 40C^g apHLAII-HA was found to significantly increase levels of IgG, as compared to non-targeted controls (aNIP-HA and HA) (Fig. 5d). The increase was evident already at day 7, and continued to increase over the next two weeks. Pigs received a second immunization with DNA/electroporation 3 weeks after the first vaccination. The boost greatly enhanced IgG levels in sera. While the first

immunization with aNIP-HA failed to induce significent levels of antibodies in sera, the second vaccination increased antibody responses. By contrast, the second immunization did not increase antibody responses to a similar extent in pigs receiving HA alone. Thus, it seems that the bivalent structure of the targeted vaccine positively contributes to the observed immune responses. Importantly, the first immunization with apHLAII-HA was found to be sufficient for induction of neutralizing antibodies (Fig. 5e), which would indicate induction of protective immunity even at this early stage.

Comparison of electroporation and jet delivery after DNA vaccination in pigs

The device (Dermavax, Cellectis) which we used for skin electroporation is approved for human use, and represents a well-tolerated system for human vaccination. However, a drawback is that DNA needs to be injected i.d. with a needle and syringe prior to electroporation, and as such presents an obstacle to prophylactic mass vaccination. Thus, we decided to also explore jet delivery of DNA. By jet delivery, drugs are propelled into the skin by high pressure with a handheld mechanical device(42), and the procedure is essentially pain-free.

Pigs were immunized with titrated amounts of aHLAII-HA plasmids, delivered either by jet delivery (Tropis, PharmaJet) or i.d. injection/electroporation (Dermavax, Cellectis). The plasmids were suspended in a 100 μĩ volume with NaCl. Animals were boosted 3 weeks after the first injection with the same amount of DNA/method, and immune responses monitored by longitudinal measurements of anti-HA IgG in sera (Fig. 6a). Results showed that a single vaccination with 100μg of apHLAII-HA induced high levels of antibodies after a single vaccination, and that there were no significant magnitudal differences between jet delivery and injection/electroporation. There was a tendency that responses after jet delivery were slightly higher, but the difference was not significant. This tendency also held true after the second vaccination, but both delivery methods greatly enhanced the induced antibody responses.

Interestingly, delivery of 25 μg apHLAII-HA was found sufficient for induction of high antibody levels when delivered twice. These results indicated that in large animals there may be an important dose sparing effect associated with targeting of antigen to MHCII molecules, and that painless and needle-free jet delivery of plasmids may substitute for needle-dependent injection of DNA and electroporation. Both these effects may be of particular importance in the developing world. Firstly, because the dose sparing effect indicates that sufficient amounts of vaccines could be available for the population at large, and, secondly, by allowing for simple, hy genie and painfree vaccine delivery.

MHCII-targeting reduces the amount of DNA needed for efficient vaccination of pigs

Both for EP and jet delivery, there was no significant difference in antibody levels elicited by either 40C^g or l OC^g of DNA. A single delivery of 25 μg DNA resulted in smaller but detectable IgG levels, and the boost increased the anti-HA Ab levels to levels similar to that obtained with either l OC^g or 40C^g of DNA. A more qualitative assessment of the induced antibodies by micro-neutralization assays demonstrated that a single immunization with lOC^g or 40C^g aHLAII-HA (but not 25μg) was sufficient for induction of neutralizing antibodies (Fig. 6b). In correspondence with results from the ELISA assay, titers of neutralizing Abs were enhanced after the boost, and even 25 μg of plasmids encoding aHLAII-HA sufficed for induction of neutralizing antibodies (Fig. 6c).

Since the above experiment did not contain non-targeted controls, we did a new experiments where targeted DNA vaccines (apHLAII-HA and aHLADR-HA) were compared with non-

targeted vaccines (aNIP-HA and HA alone), employing jet delivery. A single immunization with 75 μg DNA of either apHLAII-HA or aHLADR-HA significantly enhanced IgG levels above that observed for the non-targeted controls, as detected by ELISA (Fig. 6d). A second vaccination on day 28 further boosted responses. Where vaccination with HA alone again failed to significantly improve responses after vaccination, aNIP-HA induced high levels after the second vaccination. This result points towards an added effect from bivalently displayed antigens.

The results obtained with ELISA were corroborated by measurements of neutralizing antibodies in sera after both the first and the second immunization (Fig. 6e-f). Importantly, a single vaccination with apHLAII-HA or aHLADR-HA was sufficient for induction of protective levels of antibodies. The observed differences between MHCII-targeting and the non-targeted controls are consistent with previous observations in mice(23), and indicate that the bivalent vaccine structure in itself enhances immune response, as compared to vaccinations with antigen alone. Moreover, and importantly, these results indicate that a DNA encoded MHCII-targeted vaccine protein can induce clinically relevant antibody responses in 16-30kg animals, with relatively low amounts (75 μg) and volumes (IOOμI) of DNA, even after a single immunization. Discussion:

DNA vaccination is generally hampered by low immunogenicity, and several injections of large DNA doses are typically required for induction of protective immune responses in larger animals. Here, we have demonstrated that DNA vaccines encoding bivalent fusion proteins that target antigen to MHCII molecules on APC, enhances antibody responses in larger animals such as ferrets and pigs; a single immunization was shown sufficient for induction of antibodies with responses reaching the protective threshold for neutralizing antibodies (Fig. 6e). This may be of great importance if attempting to control an emerging influenza pandemic or other disease outbreak. Antibodies represent a strong correlate of protection against many infectious diseases, and the early antibody formation seen after MHCII-targeting could be useful in many disease scenarios.

While a single immunization with apHLAII-HA or aHLADR-HA could induce protective levels of antibodies in sera, the efficacy of the non-targeted control, aNIP-HA, was dependent on two successive vaccinations. We also included HA alone as an additional non- targeted control in several experiments. Our data indicated that vaccination with HA alone for protective efficacy would require multiple rounds of immunization, higher DNA doses per round, or the inclusion of

an adjuvant. Thus, it seems that the bivalent presentation of antigen, as conferred by the dimerization unit of our vaccine format, in itself may add to the observed immunogenicity. This effect is best explained by the bivalent antigen display allowing some cross-linking of B cell receptors, even in the absence of MHCII-targeting. Nevertheless, our data clearly points to the effect of MHCII-targeting as the decisive factor for strong induction of immune responses, but where antigenic bivalency may confer some improvement of antigen immunogenicity.

It has been suggested that the increased antibody responses observed after MHCII- targeting of antigen may be mediated by vaccine proteins bridging a synapse between APC and B cells(24,45). In such an event, the antigen could bind to B cell receptors (BCR) while the targeting unit secured interaction with the APC, allowing for efficient endocytosis of vaccine antigens and presentation on MHC class II molecules. Thus, in conjunction with T cell help, this can cause efficient stimulation of B cells and increased antibody responses. In the present work, we have not investigated the contribution of T cell responses to formation of immunity.

However, we have previously demonstrated in mice that T cells are necessary for formation of antibody responses, and that they can contribute protection in the absence of antibodies(23).

A key feature for a vaccine that is designed to bind human HLAII molecules, is that it efficiently can bind most, if not all, HLAII alleles that are found in the human population. The three most common HLAII alleles are DR2b (DRB1* 15:01), DQ2.5

(DQA1 *05:01/DQB1 *02:01) and DP4.1 (DPA1 *01 :03/DPB1 *04:01), of which the latter is expressed in about 70% of Caucasians. Importantly, there is good correlation between the binding profiles of the HLAII- and HLADR-targeted vaccines, and HLA II prevalence in the human population. OHLADR-CKCK bound all HLA DR molecules tested (13), whereas aHLAII- CKCK bound all but two HLA DR molecules (DR1104 and DR1101). DR1104 has a frequency of 0,01780 in the population, and is almost exclusively found in the region around South-Eastern Europe, whereas DRB1 *11 :01 has a frequency of 0,05945 and typically will be found in

Southern Africa or Western Asia(46). However, individuals with these alleles will also display HLA-DQ and HLA-DP molecules that are very likely to be bound by OIHLAII-CKCK (ex.

DP0101 or DP0401). In conclusion, the binding profiles of the pHLAII- and HLADR-targeted vaccines indicates that these vaccines find use for vaccinations of the human population.

Previously, we have delivered DNA by intradermal injection immediately followed by skin electroporation to enhance cellular DNA uptake(47,48). While this procedure is approved for use in humans, and typically by vaccinees described as "well tolerable", it represents a barrier towards prophylactic immunization of humans. On this background, we compared vaccine delivery by skin injection/electroporation to jet delivery in pigs. Intradermal jet delivery is a pain and needle free delivery method. Results showed that the two methods of delivery can induce similar immune responses in pigs, with a slight improvement in favor of jet delivery. Thus, we have shown that targeting of antigen to human HLAII molecules is a feasible strategy, both in terms of increased immunogenicity conferred by targeting of antigen to MHCII- molecules, and rational vaccine delivery.

Example 2

Based on the rapid spread and devastating impact of the recent Zaire ebola virus (ZEBOV) outbreak, vaccination is considered to be a crucial control measure against future occurrences of this hemorrhagic disease. Humoral immune responses have been shown to be important for vaccine-mediated protection (51), though it is understood that cell-mediated immunity may also play a role, either directly (53) or indirectly by facilitating long-lived protection against viral infection (49). The DNA vaccine platform offers several benefits concerning EBOV vaccination, including the strong induction of CD4+ T cells (52) and the potential to offer a safe priming option to be used in combination with other vaccine platforms (50). DNA vaccines based on the glycoprotein (GP) of EBOV and Marburg virus were recently demonstrated to be safe as well as induce some humoral immune responses following three vaccinations in Ugandan participants; however, no GP -specific antibodies were detected in approximately half of the participants in this phase lb clinical trial (50). Here, we tested the immunogenicity of a Vaccibody vaccine construct based on ZEBOV-GP administered intramuscularly by electroporation or needle-free jet injection in guinea pigs. We found that regardless of administration method, the Vaccibody ZEBOV-GP construct induced GP-specific IgG and IgA serum antibody responses already two weeks after one immunization in guinea pigs, and that these levels remained high at least six weeks after the second vaccination.

Although the neutralizing capability of these antibodies remains to be determined, the speed and duration of this antibody response is encouraging. Furthermore, GP-specific T cell proliferation was detected at 10 weeks following the second vaccination in both groups. Taken together, the Vaccibody ZEBOV-GP construct is a promising vaccine candidate against ZEBOV infection with potential to be administered under field conditions using a needle-free jet injector.

Methods

Animals

Twelve four to six week old female outbred Hartley guinea pigs purchased from Harlan Laboratories (Horst, Netherlands) were randomly divided into two groups of six guinea pigs each and housed in pairs at the Laboratory for Experimental Biomedicine (University of Gothenburg, Sweden). All animals were given free access to pellets and water containing Vitamin C, as well as received daily treats including crisp bread, lettuce, and carrots.

Immunizations

Before vaccination, guinea pigs were anesthetized with isofluorane (Abbott Laboratories Ltd, England) through a face mask according to standard protocols. Animals were immunized with aHLAII-GP twice at a three weeks interval, using either electroporation (group A) or the PharmaJet Stratis® needle free jet injector (PharmaJet, USA; group B). All animals were immunized in the quadriceps muscle of each leg and monitored for local inflammation for three days following each immunization.

Sample collection

Blood samples for serum isolation were collected from the saphenous veins of all animals at six time points: day 0 (before first immunization), day 14, day 21 (before second immunization), day 28, day 50, and day 63, and day 88 (before euthanization). Sera were isolated using a standard double centrifugation protocol and stored at-20°C until use. Blood samples for peripheral blood mononuclear cells (PBMCs) were collected on D91 by cardiac puncture of anesthetized animals.

Saliva samples were collected from all animals at D14, D21, and D91 using Weck-Cel® eye spears (Beaver-Visitec International, USA). Briefly, 200 μΐ of saliva isolation buffer, consisting of 1.5 g NaCl and 10 mg aprotinin in 100 ml PBS, was pipetted onto the cellulose acetate filter

of 0.22 μιτι Corning® CoStar® Spin-X® centrifuge filter tubes. Eye spears were either placed in the guinea pig mouth and held in place for 10 seconds (D14) or the guinea pig mouths were swabbed briefly while animals were under anesthesia (D21, D91), then immediately placed into SIB and stored on ice. Samples were centrifuged at +4°C for 15 min at 5000xg, the spears and filters removed, and stored at -20°C until use.

Lung supernatants were obtained from saponin-treated lung tissue samples. Briefly, following euthanization lungs were perfused with PBS, then removed from each animal and placed in 5.6 ml inhibition buffer and stored on ice. BSA (1%) was added to each lung before storage at-20°C. Saponin (10%) was added to each tube and the lungs were stored overnight at +4°C. The next day, the supernatants from all samples were relocated to 10 ml tubes, centrifuged at 4400xg for 10 min, and the supernatant stored at-20°C until use.

GP specific IgG antibody assessment

An in-house CHO cell -generated His-tagged GP of ZEBOV was used to develop an in house GP specific ELISA. Sera collected at different time points after first and second immunization were subjected individually to the GP-specific ELISA to determine specific IgG antibody titers to ZEBOV-GP. Briefly, 96-well Maxisorp ELISA plates (Nunc, Denmark) were coated overnight at +4°C with 1 μg/ml EBOV-GP, then incubated for 60 min at room temperature with 1% BSA+PBS-Tween following a PBS wash. The plates were washed with PBS -T ween, incubated for 90 min at room temperature with seven three-fold dilutions of guinea pig sera (1 : 10 to 1 :7290) in blocking buffer, then washed before a final incubation with HRP- conjugated secondary antibody (goat-anti guinea pig IgG, 1 :6000 dilution) for 90 min at room temperature. Results were visualized using TMB peridoxase substrate (KPL, USA), stopped with 1M H2S04, and absorbance measured at 450nm. Results are expressed as loglO- transformed antibody titers, calculated by linear regression to the negative cut off, defined as the average OD value of blank wells plus 3 times the standard deviation of those blank wells.

GP -specific IgA antibody assessment

An in house CHO cell generated His tagged ZEBOV GP was used to develop an in house GP specific ELISA. Sera, saliva, or lung supernatants collected at different time points were subjected individually to the GP specific ELISA to determine specific IgA antibody titers to

ZEBOV GP. Briefly, 96 well Maxisorp ELISA plates (Nunc, Denmark) were coated overnight at +4°C with 1 μg/ml ZEBOV GP, then incubated for 4 h at room temperature with 5% skim milk in PBS Tween following a PBS wash. The plates were washed with PBS Tween, incubated for 2 h min at room temperature with 4 6 three fold dilutions of guinea pig sera, saliva, or lung supernatants (1 : 10 to 1 :2430; 1 :5 to

1 : 135; and 1 :5 to 1 : 1215, respectively) in blocking buffer, then washed before a final incubation with HRP conjugated goat anti guinea pig IgA (1 :4000 dilution; AbDSerotec, UK) for 90 min at room temperature. Results were visualized using TMB peridoxase substrate (KPL, USA), stopped with 1M H2S04, and absorbance measured at 450nm. Results are expressed as loglO transformed antibody titers, calculated by linear regression to the OD value of the previously defined negative cut off.

ZEBOV GP specific PBMC proliferation analysis

Blood samples for PBMC isolation was collected using a 10 ml syringe into individual 50 ml tubes containing heparin, then immediately diluted 1 : 1 with room temperature PBS. PBMCs were isolated by FICOLL Paque gradient and washed twice in PBS before diluting in complete Iscove's medium (Life Technologies, USA) including 10% fetal bovine serum and 1% each of β mercaptoethanol, L glutamine, and gentamicin. Cells were counted, plated in flat bottomed 96 well plates at 200,000 cells/well, and stimulated in triplicate with either 4 μg/well ZEBOV GP, 5 μg/well Concavalin A (ConA), or medium alone. Plates were incubated at 37°C with 5% C02 for 96 h, then 60 μΐ of supernatant removed per well and 20 μΐ of thymidine, diluted 1 :20 in complete Iscove's medium, was added to each well and incubated for an additional 7 h at 37°C. Cells were harvested using a Cell Harvester (Tomtec, USA) and counted using a MicroBeta plate counter (Perkin Elmer, USA) according to standard protocols. Stimulation indices indicating fold changes of PBMC proliferation compared to medium alone, were determined by dividing the average corrected count per minute (CCPM) of GP or ConA stimulated wells by that of wells containing no stimulant. Only samples with an average CCPM greater than 6000 in ConA stimulated wells were included in GP specific PBMC proliferation analyses.

Statistics

Two-way ANOVA tests or two tailed t tests were performed among groups at the different time points or between groups at the same time point, using Prism 6.0 (GraphPad, USA), to determine statistical significance based on ap value less than 0.05.

Results

HLAII-targeted GP construct elicits strong ZEBOV GP specific IgG serum antibody responses by both electroporation and jet injection ZEBOV GP specific IgG antibody responses were detected in the sera of 5/6 immunized animals in group A and 6/6 immunized animals in group B two weeks after the first immunization (day 14, Figure 7). One week following the second immunization, specific antibodies were detected in the sera of all immunized guinea pigs at levels that were significantly higher in group A compared to group B. However, by day 50, no significant differences were observed between groups and antibody levels remained elevated at least through day 63 (last time point tested), at approximately 5 loglO titer for both groups.

HLAII-targeted GP construct induces long lasting ZEBOV GP specific systemic, but not mucosal, IgA antibody responses by both electroporation and jet injection

Sera, saliva, and lung supernatants from all vaccinated animals were collected and subjected to an in house ELISA, to determine systemic (serum) or mucosal (saliva, lung supernatants) IgA antibody responses following vaccination with the HLAII-targeted GP construct. IgA antibody responses in serum increased in all vaccinated individuals at two weeks after the first vaccination (day 14, Figure 8 A) and continued to increase one week after the second vaccination in both groups at levels that were statistically significant compared to day 0 ($p<0.0001$ for both groups). These GP specific antibody titers peaked at day 28 at approximately 2 loglO titer. At five weeks following the second vaccination (day 63), levels had decreased compared to day 28, but remained statistically significant compared to day 0 (pO.001) in both groups. ZEBOV GP specific IgA antibodies were also detected at low levels in saliva samples collected from vaccinated guinea pigs (Figure 2B), but not in lung supernatants (data not shown). These levels were highest at two weeks after first vaccination (day 14) and were significant in the electroporation group compared to negative controls at this time point ($p\leq0.01$), but decreased before second vaccination and remained at similar levels to the negative controls at the end of the study (day 91).

HLAII-targeted GP construct stimulates specific PBMC proliferation by both electroporation and jet injection

PBMCs isolated from vaccinated guinea pigs were stimulated for 96h with ZEBOV GP and their proliferation measured using a 3H thymidine based assay in comparison with medium alone. Both groups of vaccinated animals showed a marked increase in PBMC proliferation following GP stimulation, which averaged 6 fold and 11 fold higher than medium alone in the electroporation and jet injection groups, respectively. Two of 6 individuals in the electroporation group and 3/6 in the jet injection group were excluded from analyses based on low relative proliferation following ConA stimulation, and therefore a small number of animals were included in the analysis. However, the same relative pattern of GP specific proliferation (higher in the jet injection group than the electroporation group) is shown with all data points included. Therefore, although these results were not statistically significant, they suggest that the jet injection method may induce greater PBMC proliferation following GP specific stimulation compared to the electroporation administration.