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Research paper

Vaccination with influenza hemagglutinin-loaded ceramic nanoporous microneedle arrays induces protective immune responses

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ABSTRACT

Microneedle arrays (MNAs) are a promising mean to administer vaccines. Without the need of highly trained personnel, MNAs can be applied to deliver vaccines into the dermis, which is well equipped to initiate potent immune responses. While vaccination using dissolving microneedle arrays has been extensively investigated, the use of solid nanoporous MNAs (npMNAs) to deliver vaccines remained largely unexplored. In this report we investigated whether npMNAs with an average pore size of 80 nm, can be used for influenza vaccination based on recombinant hemagglutinin (HA) protein of the 2009 pandemic H1N1 (pH1N1) virus. Fluorescently labeled HA loaded in the npMNAs was effectively delivered into the skin of mouse ears, as a result of a diffusion-based process. Compared to intramuscular immunization, intradermal HA vaccination of mice using npMNAs elicited high levels of HA antigen specific antibodies, with pH1N1 hemagglutination inhibition and neutralization activity. Moreover, mice vaccinated with pH1N1 HA loaded npMNAs were completely protected against a potentially lethal challenge with mouse adapted pH1N1 virus. These results illustrate that intradermal subunit vaccine immunization using npMNAs is a promising approach to facilitate effective vaccination.

1. Introduction

Influenza A and B viruses are a major threat to the public health and influenza A viruses have repeatedly caused pandemics. Vaccination is considered a cost-effective measure against influenza [1]. However, current licensed human influenza vaccines provide strain-specific protection due to the constant antigenic drift of circulating seasonal influenza viruses. Furthermore, influenza A viruses can also undergo antigenic shifts, whereby a novel influenza virus enters the human population, spreads and causes a pandemic. As such, the majority of the human population lacks significant pre-existing immunity against a new pandemic virus resulting in wide-spread infection, morbidity and mortality. For the vaccine manufactures, a pandemic influenza A virus outbreak, such as the Mexican flu that emerged in 2009, represents a formidable challenge because massive amounts of a new vaccine have to be prepared and distributed in a short period of time [2,3]. Therefore, preparedness for seasonal as well as pandemic influenza outbreaks requires regular massive vaccination campaigns. Licensed influenza vaccines require cold chain logistics and the vast majority of marketed influenza vaccines are filled in a needle-and-syringe, which requires trained personnel to perform the vaccination. Intramuscular injection of influenza vaccines is often associated with transient discomfort such as intramuscular pain, occasional needle-stick injury and may reduce influenza vaccine use by individuals with needle phobia. The use of microneedle arrays (MNAs) for intradermal vaccination may circumvent some or all of these issues. Dry antigen formulations, for example antigens coated onto MNA surfaces or antigens entrapped in dissolving MNAs, can remain stable at room temperature and allows for self-vaccination [4–6]. Moreover, microneedle-based influenza vaccines have the potential to be more immunogenic than standard intramuscular immunization [7]. While dermal vaccination by the use of coated and dissolving MNAs has been extensively studied, the use of

Abbreviations: AlOH, Alum; DC, dendritic cells; DT, diphtheria toxoid; HA, hemagglutinin; HAI, hemagglutination inhibition; Im, Imiquimod; IM, intramuscularly; npMNA, nanoporousmicroneedlearray; (pH1N1), 2009 pandemic H1N1; SAS, Sigma Adjuvant System; SC, subcutaneous; TT, tetanus toxoid; TLR7, TollLikeReceptor7

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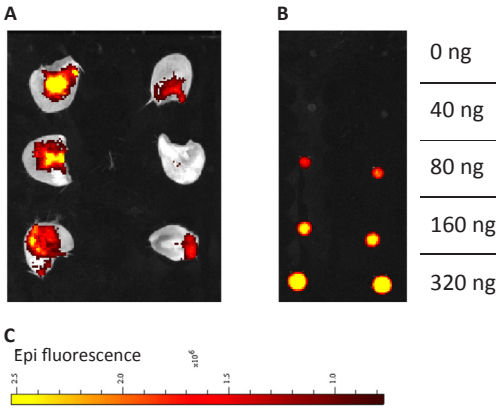

Fig. 1. Delivery of hemagglutinin (HA) into *ex vivo* mouse ears by nanoporous microneedle arrays (npMNAs). IRdye800cw labeled HA (0.8 μg/μL) was loaded into npMNAs and applied to mouse ears *ex vivo*. (A) IRdye800cw fluorescence of *ex vivo* mouse ears into which HA was delivered by npMNAs (left, n = 3) and untreated control ears (right, n = 3). The numbers represent the level of IRdye800cw fluorescence of the indicated regions of interest. (B) IRdye fluorescence of 0, 40, 80, 160 and 320 ng labeled HA used to create a standard curve to quantify the amount of HA delivered in A. This analysis revealed that npMNAs loaded with 5 μl of 800 ng/μl HA deliver about 427 +/- 101 ng HA to the dermis of mice.

nanoporous MNA (npMNA) mediated vaccination obtained little attention in literature. The goal of the present study was to investigate the immunogenicity and protection of intradermal vaccination with a recombinant influenza hemagglutinin (HA) antigen using npMNAs.

2. Results

2.1. HA antigen is effectively released in ex vivo mouse ears by npMNAs

To determine the amount of HA antigen that can be delivered to the ears of mice using npMNAs, fluorescently-labeled HA antigen loaded into npMNAs were applied to the ears of mice *ex vivo* (Fig. 1). HA was effectively released from the npMNAs into the ear dermis (as a result of diffusion), as determined by IR imaging (Fig. 1A). The exact amount of HA loaded into the npMNAs could not be determined (only roughly estimated). Furthermore, the residual amount of HA in the npMNAs could not be determined, because the npMNAs are not transparent for the wavelengths used to detect the fluorophore. Therefore, in this study only the amounts that were released into the mouse ears were determined. The release of HA into the mouse ears was quantified by using a standard curve (Fig. 1B) and correcting for the background fluorescence of untreated control ears. This revealed that npMNAs loaded with a concentration of [*X* ng HA per μL] results in a delivery of [53% of *X* ng HA antigen] after 30 min of application.

2.2. Dermal vaccination with HA-loaded npMNAs elicits HA-specific serum IgG responses

To test whether npMNAs can be used to deliver antigens for vaccination, npMNAs were loaded with different amounts of recombinant influenza pH1N1 HA protein and applied to the ears or the lower back of BALB/c mice. As controls, mice were mock immunized with PBS using npMNAs (negative controls), immunized intramuscularly with HA combined with or without Alum (AlOH), or subcutaneously with HA in combination with the Sigma Adjuvant System (SAS) (positive controls). All animals were primed followed by a booster immunization. To test HA antigen-specific antibody responses, sera were collected before and after each immunization. All mice that were immunized with HA by intramuscular injection and all mice that were immunized with HA in combination with SAS adjuvant by subcutaneous injection had high levels of HA-specific serum IgG (Fig. 2A). HA-specific IgG could also be detected in all mice that had been immunized with microneedle arrays applied to the ears (Fig. 2A and B). In contrast, application of HA-loaded microneedle arrays to the lower back did not result in robust serum IgG responses. Addition of the TLR7 agonist imiquimod to the HA antigen that was administered via microneedle arrays did not significantly enhance the IgG response. Seroconversion of the mice that had been immunized with npMNAs applied to the ears was antigen dose dependent (Fig. 2A). Immunization with 3 μg HA by applying npMNAs to the ears elicited modestly higher serum anti-HA IgG as well as HAI titers as compared to intramuscular immunization (Fig. 2A and C, respectively). To test the functional activity of the HA-specific antibodies induced by immunization via the different routes, hemagglutination inhibition experiments were performed. Sera of mice that had been immunized with 3 μg HA by intramuscular injection or by applying npMNAs to the ears displayed pH1N1 virus hemagglutination inhibition (HAI) activity (Fig. 2C). In line with this, sera from mice that had been immunized with 3 μg HA via npMNAs could also potently prevent pH1N1 infection *in vitro* (Fig. 2D). These data suggest that vaccination with recombinant HA antigen using npMNAs could provide protection in mice against pH1N1 infection.

2.3. Dermal HA vaccination elicits protective immunity against homologous influenza A virus infections

To test whether HA immunization using microneedle arrays can protect against pH1N1 infection, mice were immunized twice with 3 μg HA via npMNAs applied to the ears or via intramuscular injection and subsequently challenged with a dose of pH1N1 that was lethal for negative control treated mice. As expected, all mice that were immunized with HA had high levels of HA-specific serum IgG. Mice that were immunized using npMNAs had moderately higher titers of anti-HA IgG than mice immunized IM (Fig. 3A–C). Importantly, HA immunization using npMNAs elicited both IgG1 and IgG2a subclass antibodies (Fig. 3D–F). HA immunization using npMNAs induced HAI antibodies to a comparable extent as intramuscular immunization (Fig. 4A). In addition, serum of individual mice that had been immunized with HA-loaded npMNAs could effectively neutralize pH1N1 *in vitro* (Fig. 4B). Intramuscular immunization of HA with or without Alum adjuvant also induced pH1N1 neutralizing antibodies but not in all mice (Fig. 4B). To test whether vaccination with HA-loaded microneedles can protect against infection, the mice were challenged with a potentially lethal dose of pH1N1 and subsequently monitored for bodyweight change and mortality (Fig. 4C and D). As expected, PBS-immunized mice rapidly lost bodyweight and all animals in this group had to be euthanized per protocol by day eight. In contrast, all HA-vaccinated mice survived the infection without substantial body weight loss. These data illustrate that HA vaccination using npMNAs induces HA-specific antibody responses that correlate with protection of mice against challenge infection with a homologous influenza virus.

3. Discussion

While the outer layer of the skin, the stratum corneum, is an important physical barrier, the underlying viable epidermis and dermis form an immunological interface containing various leukocytes including antigen presenting epidermal dendritic cells (DCs) (Langerhans cells) and dermal DCs [8]. These DCs can take up antigens in the skin, migrate to the skin draining lymph nodes to activate naïve T cells. This makes the skin an attractive site for immunization, which has been intensively explored for improvement of influenza vaccination [7,9]. Protection against seasonal influenza epidemic requires annual vaccination demanding fast production of high amounts of vaccine antigen. This is also required in case of a new influenza pandemic. Intradermal vaccination has been explored as a means for vaccine antigen dose sparing. Multiple clinical trials have demonstrated that intradermal

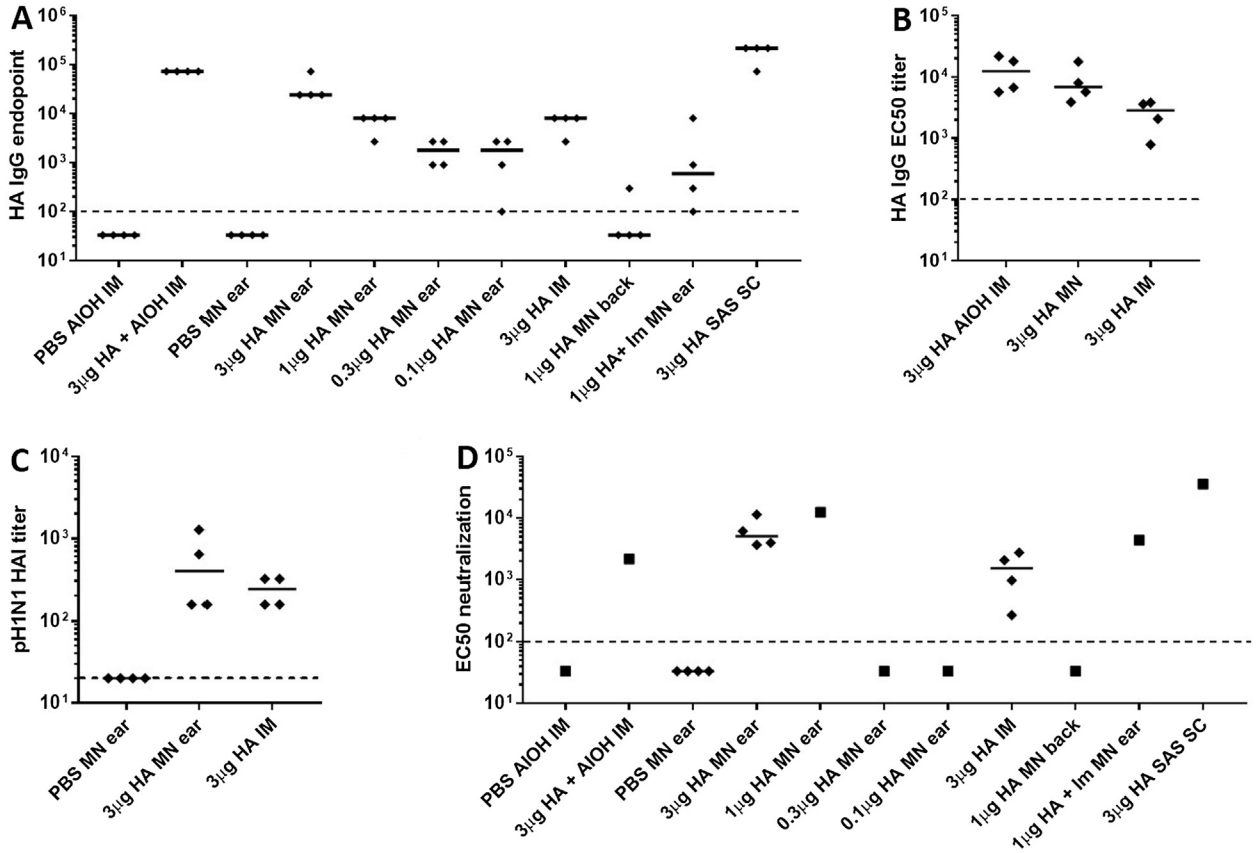

Fig. 2. Dermal HA vaccination using nanoporous microneedle arrays (npMNAs) induces hemagglutinin (HA)-specific IgG that can block pH1N1 hemaggultination and infection. Groups of four mice were vaccinated twice with PBS or 3, 1, 0.3 or 0.1 μg HA via the intramuscular (IM), subcutaneous (SC) or intradermal (microneedle mediated (MN)) route either in the presence or in the absence of Alum (AlOH), imiquimod or the Sigma Adjuvant System (SAS). Microneedle arrays were either applied onto the ears or onto the back. Serum samples were collected before each immunization and 18 days after the last immunization. The anti-HA IgG titers were determined by HA protein ELISA. (A) Anti-HA IgG endpoint titers present in serum of mice of the indicated groups 18 days after the second immunization. The symbols and lines respectively represent the individual and median anti-HA IgG endpoint titers. (B) Anti-HA IgG EC50 titers present in serum of mice of the indicated groups 18 days after the second immunization. The symbols and lines respectively represent the individual and median anti-HA IgG Anti-HA IgG EC50 titers. (C) Hemagglutination inhibitory (HAI) antibodies in serum of mice after boost vaccination with HA or PBS via microneedle arrays or intramuscular injection. The symbols and lines represent the individual and median HAI titers (n = 4), respectively. (D) Virus neutralizing activity present in serum of mice 18 days after the second immunization. The single symbols represent the EC_{50} neutralization titers of pooled sera per group. For mice immunized with PBS or 3 μg HA via npMNAs and mice immunized with 3 μg HA via intramuscular immunization the individual sera were tested and shown as symbols whereas the line represents the median (n = 4). In each graph the dashed line indicates the limit of detection.

vaccination with a reduced antigen dose (3, 6 or 9 μg HA per strain) is either non-inferior or even superior to conventional IM or SC vaccination using the conventional full dose of 15 μg HA per strain [10–12]. Intradermal vaccination can be performed by experienced staff using a conventional needle (Mantoux method) or can be applied in a more practical way by the use of novel devices designed for intradermal vaccination such as the BD 1.5 mm Soluvia needle or the 450 μm or 600 μm MicroJet microneedles. Low dose ID influenza vaccinations using such a device (Soluvia needles) was more efficient than low dose ID vaccination using a conventional needle or than IM vaccination using the standard 15 μg HA dose [13]. Although seasonal influenza vaccination is usually effective in healthy adults (up to 70% protection may be achieved), it is much less effective and heavily debated in elderly [14]. Intradermal vaccination of elderly with the standard HA antigen dose has been shown to be remarkably more immunogenic than conventional IM vaccination using the same antigen dose [15]. The immunogenicity of ID influenza vaccination could be further enhanced by topical application of imiquimod, which resulted in significantly reduced hospitalization rate for pneumonia or influenza as compared to volunteers immunized IM [16]. Recently, a phase 1 clinical trial demonstrated that an inactivated influenza vaccine delivered into the skin by dissolvable microneedle patches is safe and highly immunogenic [6]. In light of these results, there is a strong interest in novel, easy to apply methods that can specifically deliver vaccine antigens in the dermis that contains high levels of APCs [7].

The ceramic npMNAs that were used in this study contain 105 microneedles, each with a length of 475 μm. Although those microneedles have a porosity of 40% and an average pore diameter of 80 nm) they have sufficient strength to penetrate the stratum corneum allowing for diffusion of the vaccine antigen out of the nanopores into the dermis. Previously it was shown that diphtheria toxoid (DT) and tetanus toxoid (TT) subunit vaccination using npMNA can induce antigen specific antibodies [17]. In this paper we report that vaccination with recombinant HA loaded into npMNA can induce influenza neutralizing antibodies and protect mice from a potentially lethal influenza virus infection.

In contrast to conventional (hypodermic) needles and hollow microneedles, antigen loaded into npMNAs need to diffuse passively from the npMNA pores into the dermis. Our results demonstrate that npMNAs can effectively deliver HA antigen into the skin of mouse ears and can effectively induce HA-specific antibody responses. Moreover, the levels of serum anti-HA IgG elicited by intradermal immunization using npMNAs were modest but significantly higher than those induced by IM immunization using a similar dose. This is in line with

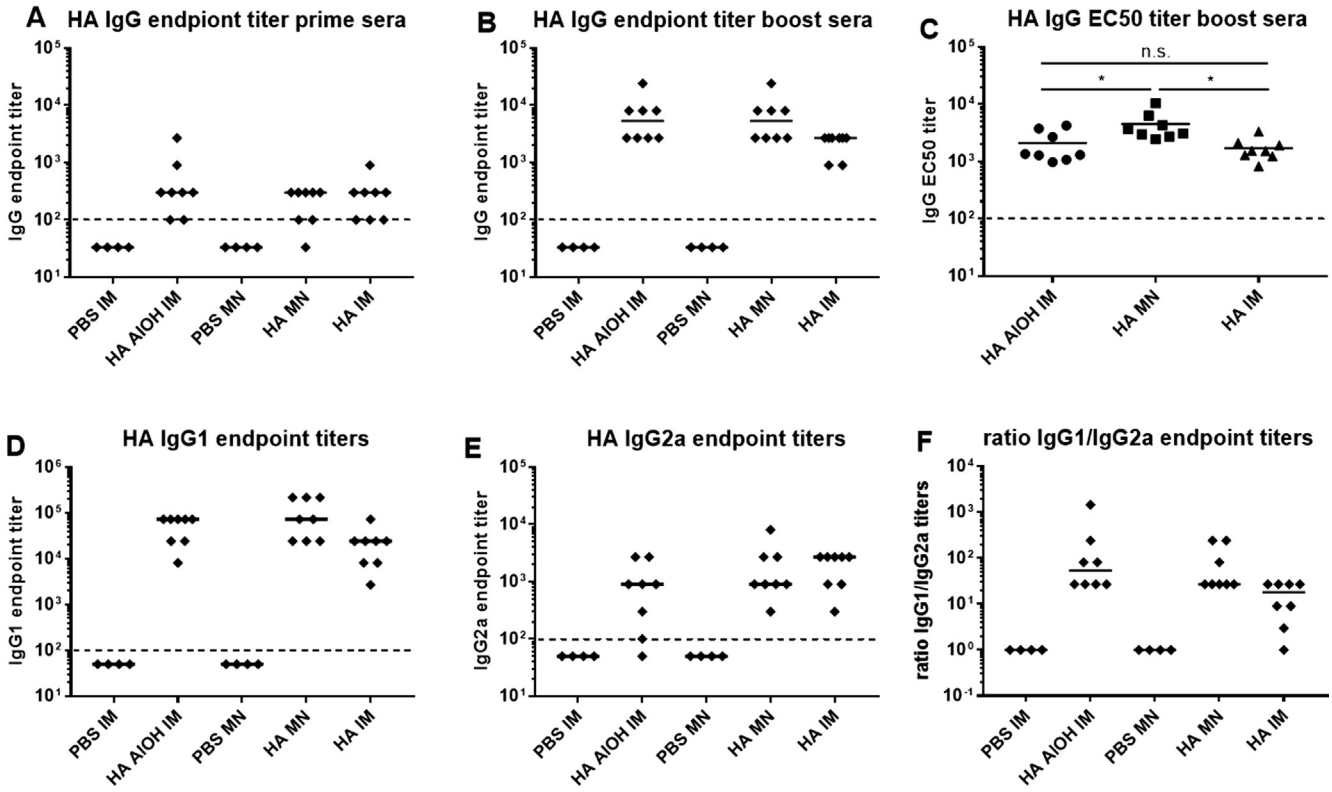

Fig. 3. HA immunization via nanoporous microneedle arrays (npMNAs) induces both IgG1 and IgG2a anti-hemagglutinin (HA) antibodies. Groups of four or eight mice were vaccinated twice with the indicated vaccines and through the indicated routes. Serum samples were collected before each immunization and 18 days after the last immunization. The anti-HA IgG titers were determined by HA protein ELISA. (A and B) Anti-HA IgG endpoint titers present in serum of mice of the indicated groups 18 days after the first (A) and second immunization (B) (IM = intramuscular injection, MN = dermal vaccination using microneedle arrays applied to the ears). The symbols and lines represent the individual and median anti-HA IgG endpoint titers, respectively. (C) Anti-HA IgG EC_{50} titers present in serum of mice of the indicated groups 18 days after the second immunization. The symbols and lines represent the individual and median anti-HA IgG Anti-HA IgG EC50 titers, respectively. The EC_{50} titers of anti-HA serum IgG in mice immunized with HA using microneedle arrays were significantly higher than those of mice immunized via intramuscular injection ($p < 0.05$, 1-way ANOVA, Dunn s multiple comparison test). (D and E) Anti-HA IgG1 (D) and IgG2a (E) endpoint titers present in serum of mice of the indicated groups 18 days after second immunization. The symbols and lines represent the individual and median anti-HA IgG endpoint titers, respectively. (F) The ratio of the IgG1 and IgG2a anti-HA endpoint titers present in serum of mice of the indicated groups 18 days after second immunization. The symbols and lines represent the individual and median anti-HA IgG1/anti HA IgG2a ratios, respectively. In each graph the dashed line indicates the limit of detection.

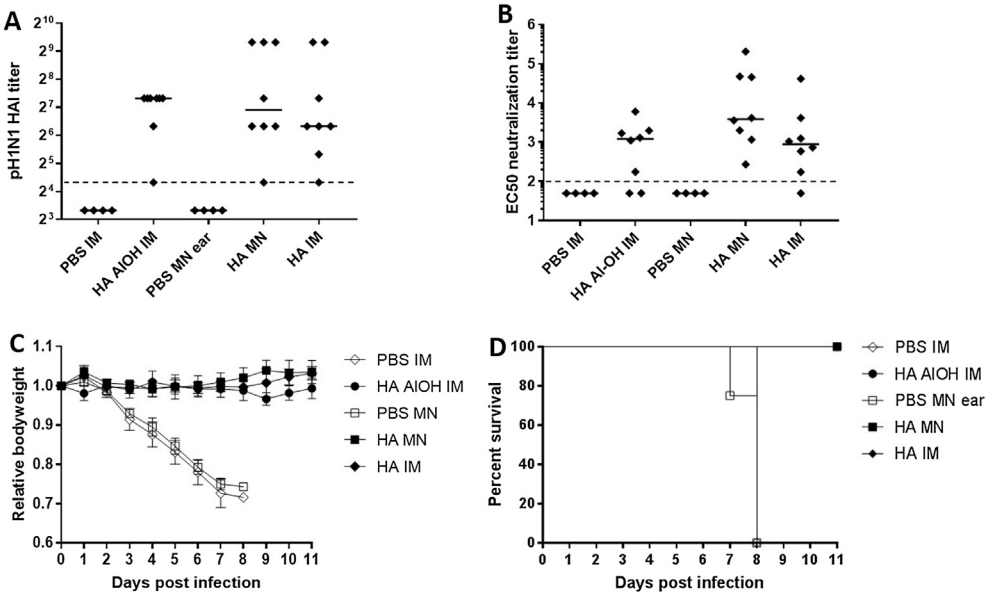

Fig. 4. Hemagglutinin (HA) immunization via nanoporous microneedle arrays (npMNAs) induces pH1N1 neutralizing antibodies and protects against pH1N1 infection in mice. (A) Hemagglutination inhibitory (HAI) antibodies in serum of mice after boost vaccination with HA or PBS via microneedle arrays or intramuscular injection. The symbols represent the serum HAI titer of each individual mouse, whereas the lines represent the medium HAI titer (n = 8). (B) Virus neutralizing activity present in serum of mice 18 days after the second immunization. The symbols represent the serum EC_{50} pH1N1 neutralization titer of each mouse, whereas the lines represent the median EC_{50} neutralization titers (n = 8). Three weeks after the second vaccination the mice were challenged with $2 \times LD_{50}$ of mouse adapted pH1N1. (C) Relative body weight of vaccinated mice after challenge. The graphs indicate the average (± S.D.) relative body weight at the indicated days after challenged mice. From 3 days onwards, mice that were immunized with HA either via intramuscular injection (IM) or via npMNAs (MN) lost significantly less bodyweight as compared to mice immunized with PBS ($p > 0{,}0001$, Two-way Anova, Tukey's Multiple Comparison test). (D) HA immunization via MNAs protects mice against lethality upon pH1N1 infection. The graph displays percentage survival. Mice that were immunized with HA via npMNAs were significantly more protected against lethality than mice that were mock immunized with PBS via npMNAs ($p > 0{,}0001$, Log-Rank Mantel-Cox test). In each graph the dashed line indicates the limit of detection.

observations for other types of microneedle patches used for intradermal influenza vaccination [18–20]. The level of induced HA-specific IgG correlated with the amount of antigen used to load the npMNAs. The highest dose of HA loaded into npMNAs was 3 μg which corresponds to the lowest dose (of each HA in tri- or tetravalent inactivated split virion vaccine) that has been administered intradermally in clinical trials and which was shown to be non-inferior to the conventional 15 μg dose used for IM vaccination. The Flublok® vaccine of Protein Sciences Corporation from which the recombinant HA protein used in this study was obtained, contains 45 μg recombinant HA of each included influenza strain [21]. It cannot be excluded that npMNA intradermal vaccination with HA doses higher than used in this study could elicit higher antibody responses. Compared to npMNA immunization at the lower back, npMNA immunization at the ears was much more immunogenic. Superior immunogenicity of vaccination at the ears has been observed before and illustrates the importance of the site of immunization [22]. We tested if the TLR7 agonist imiquimod, loaded on npMNAs together with the HA antigen could increase antibody responses. This was however not the case, but in correspondence with what was observed for ID vaccination of DT and TT using npMNAs by de Groot et al [17]. In this study it was observed that imiquimod loaded on npMNAs was only partially released, indicating that it might adsorb onto the npMNA matrix material [17]. Next to co-delivery of imiquimod with the antigen, imiquimod can also be applied topically to the skin before ID vaccination. This approach was shown to potently increase influenza vaccine responses and reduce pneumonia or influenza related hospitalizations [16]. In addition, other small adjuvants such as CpG, flaggeling and Cholera toxin that would fit in the npMNA pores could be evaluated in future vaccination experiments.

In general, it is believed that influenza vaccine induced HA antibodies control virus infection mainly by interfering with the interaction of HA with its cellular receptor, sialic acid, on the surface of the target cells. The HA-specific antibodies induced by npMNA vaccination were able to interfere with influenza virus mediated hemagglutination of red blood cells and were able to neutralize influenza in an *in vitro* plaque reduction assay. This illustrates that at least part of the HA delivered by npMNAs is intact. Next to direct neutralization, HA-specific antibodies can also employ effector functions through their Fc domain, such as complement mediated cytotoxicity, antibody-dependent cell mediated cytotoxicity or antibody-dependent cellular phagocytosis to clear infected cells or virions. In mice especially subclass IgG2a antibodies have potent Fc-dependent effector functions. We observed that the anti-HA IgG induced by npMNA vaccination consisted of both IgG1 and IgG2a subclasses. Compared to IM vaccination npMNA ID vaccination tended to be more oriented towards induction of IgG1 subclass antibodies. This is in line with what was observed for DT/TT vaccination using npMNAs [17]. Finally, we demonstrated that HA vaccination using npMNAs could completely protect against a lethal infection of an influenza strain (pH1N1 /2009) matching the HA antigen.

Several things should be considered when translating a microneedle technology for human use. Currently, the npMNA technology is being translated for human use. Although the risk of infection using npMNAs is very low (after sintering at 1450 °C the npMNAs are considered to be sterile, followed by aseptic handling, and the npMNAs have an average pore size of 80 nm, which is too small for bacteria to enter), for human studies and commercial products procedures should be in place to pack, sterilize and label npMNAs individually. Another challenge is the scale-up of npMNA production to industrial levels. The current production set-up allows to produce multiple thousands of npMNAs per year. For large scale commercial production, the current process needs to be translated into a more automated, large scale production and quality control. Finally, in this study we used a microneedle applicator that was specially designed for the usage in animal studies [17]. For the application of npMNAs by patients or their care givers, an applicator will be used as well. This is in order to assure a user independent application of npMNAs and an efficient and reproducible skin piercing. This could be an electrically-driven applicator (e.g., [23]), or an applicator for manual insertion (e.g., [24]) that contains a feedback mechanism to confirm proper application.

4. Conclusion

In conclusion, in this paper we show the applicability of the alumina npMNA technology for the induction of protective immune responses against influenza. Therefore, our results indicate that npMNA-based antigen delivery is a promising approach for intradermal vaccination against influenza and other pathogens.

5. Material and methods

5.1. Preparation and characterization of antigen loaded npMNAs

npMNAs (type 1.2.0: diameter of 9 mm, 475 μm long, 150 microneedles/cm^2, average pore size of 80 nm) were manufactured according to MyLife Technologies' proprietary production process at LouwersHanique (the Netherlands), as previously described [17].

Microneedles were pierced through a foil (Parafilm®) by using a UAFM-V1 electrical applicator (uPRAX Microsolutions) at a velocity of 65 cm/s in order to only load the microneedle tips (avoiding loading of antigen in the nanoporous backplate) as illustrated in Fig. 5. Next, a drop of 5 μl vaccine formulation was applied onto the foil-pierced npMNAs to absorb the vaccine formulation into the microneedle tips. After 5 s, the surplus drop of vaccine formulation and the foil were sequentially removed from the npMNAs [17].

5.2. Quantification of antigen release in ex vivo mouse ears

The released antigen dose was quantified by piercing npMNAs, which were loaded with IRdye 800CW (Li-Cor) labeled antigen, into *ex vivo* mouse ears, as previously described [17]. To quantify the amount of HA delivered into murine skin, npMNAs of which only the tips were loaded (using foil piercing to shield the npMNA surface in between the individual microneedles) with fluorescently labeled HA were prepared by using 5 μl of 0.8 mg/ml HA. After applying the antigen loaded npMNAs for 30 min to the ears by using a 3D-printed applicator provided by uPRAX Microsolutions, the npMNAs were removed. The fluorescence of HA delivered to the ears was measured using an IVIS® lumina II equipped with an ICG filter set and Living Image® software (version 4.3.1). The amount of delivered HA was calculated using a standard curve of known amounts of fluorescently-labeled HA and correcting for background fluorescence of untreated control ears.

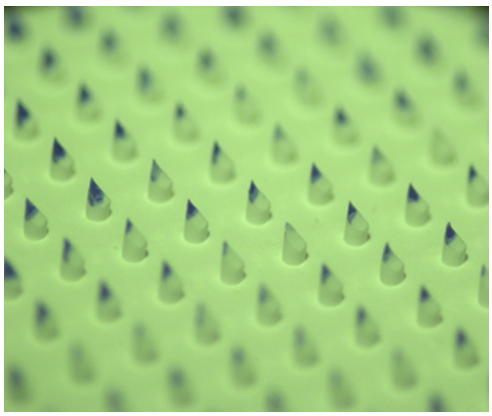

Fig. 5. Brightfield microscopy images of a nanoporous microneedle array (npMNA) of which only the microneedle tips are loaded with a trypan blue solution.

Table 1
Different groups in the two immunization studies whereby the vaccine was administered via different administration routes (intramuscular (IM), intradermal (ID) via nanoporous microneedle arrays (npMNAs), subcutaneous (SC)) and using different adjuvants (Alum (AlOH), imiquimod, and Sigma Adjuvant System (SAS)). The last two columns represent which groups were present in which of the two studies.

Administered dose HA	Administration route	Delivery device	Adjuvant	Study 1 (4 mice/group)	Study 2 (8 mice/group)
–	IM	Needle and syringe	AlOH	+	+
3 μg	IM	Needle and syringe	AlOH	+	+
3 μg	IM	Needle and syringe	–	+	+
3 μg	SC	Needle and syringe	SAS	+	
–	ID (ear)	npMNA	–	+	+
3 μg	ID (ear)	npMNA	–	+	+
1 μg	ID (ear)	npMNA	–	+	
1 μg	ID (ear)	npMNA	imiquimod	+	
0.3 μg	ID (ear)	npMNA	–	+	
0.1 μg	ID (ear)	npMNA	–	+	
1 μg	ID (dorsal (back))	npMNA	–	+	

5.3. Cells and viruses

MDCK (Madin–Darby canine kidney) cells were cultured in DMEM supplemented with 10% FCS, non-essential amino acids, 2 mM L-glutamine, 0.4 mM sodium-pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific, Massachusetts) at 37 °C in 5% CO_2. pH1N1 is derived from a clinical isolate of the pH1N1 virus of 2009 (kindly provided by Dr Isabelle Thomas, Scientific Institute of Public Health, Brussels, Belgium), and was adapted to mice by serial passages [25]. Viruses were produced in MDCK cells in serum-free medium in the presence of TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin (Sigma-Aldrich, Saint-Louis, MO).

5.4. Ethics statement

All animal experiments described in this study were conducted according to national legislation (Belgian laws 14/08/1986 and 22/12/2003, Belgian Royal Decree 06/04/2010) and European legislation (EU Directives 2010/63/EU and 86/609/EEC). All experiments on mice and animal protocols were approved by the ethics committee of Ghent University (permit numbers LA1400091 and EC2015-055). All efforts were made to avoid or ameliorate suffering of animals.

5.5. ELISA

ELISA was used to determine antibody titers in sera from individual mice. To determine HA-specific IgG in mouse sera, microtiter plates (type II F96 MaxiSorp, Nunc) were coated with 0.1 μg of purified insect cell derived recombinant HA (from A/California/07/2009, Flublok®, purchased from Protein Sciences Corporation) in 100 μl PBS (pH 7.2) (Thermo Fisher Scientific, Massachusetts) and incubated overnight at 4 °C. A peptide corresponding to the RSV A2 SH ectodomain (SLLTEVETPIRNEWGCRCNDSSD) [26] was used as negative control coated antigen. After washing with PBS + 0.1% Tween®, the plates were blocked for 1 h with 200 μl of 4% skim milk in PBS. After 1 h incubation, the plates were washed again with PBS containing 0.1% Tween® 20. A series of threefold dilutions of mouse serum samples, prepared in PBS with 2% milk and 0.1% Tween® 20, starting with a 1/100 dilution, were loaded on the antigen-coated plates and incubated for 2 h at room temperature. After washing, antigen-specific antibodies were detected by using peroxidase-labeled antibodies directed against mouse IgG, IgG1 or IgG2a (individually diluted 1/6,000) (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), or against total mouse IgG in PBS with 3% skim milk and 0.05% Tween® 20. Next, the microtiter plates were washed again and were incubated for 5 min with TMB substrate (tetramethylbenzidine, Sigma-Aldrich). The reaction was stopped by adding an equal volume of 1 M H_2SO_4, and the absorbance at 450 nm was measured. Endpoint HA-specific IgG titers were defined as the highest dilution for which a twice as high optical density (O.D.) value was measured as compared to the O.D. value of the corresponding dilution on the plate coated with RSV A2 SH ectodomain antigen. For each group in which the serum dilution series of all samples reach both the maximum and minimum O.D. value, the individual ELISA EC50 values were calculated. The HA-specific antibody EC50 titers were calculated using non-linear regression analysis in GraphPad Prism (v6.07) software for Windows (GraphPad Software, San Diego, CA). A value corresponding to 50% of the limit of detection was assigned to each sample for which no binding was detected.

5.6. Mice immunization and challenge

Specific pathogen-free (SPF) female BALB/c mice with an age of 7–8 weeks were purchased from Charles River and housed in a Biosafety level 2, SPF temperature-controlled environment with 12 h light/dark cycles and were given water and food ad libitum. The mice were let to adapt to the animal room for 1 week. The different groups that were immunized in the two immunization studies are summarized in Table 1 and the immunization, sampling, and challenge scheme is presented in Fig. 6. In the first animal study, the mice were immunized with different doses of HA, either formulated in PBS or formulated with $Al(OH)_3$ (AlOH), 1 μg imiquimod (InvivoGen, San Diego, CA) or Sigma Adjuvant Systems (SAS, Sigma-Aldrich, St. Louis, USA). For immunization via npMNAs, the indicated doses of HA were loaded into the npMNAs and subsequently applied on the back or onto both ears of the mice using a 3D-printed impact insertion spring loaded applicator (uPRAX Microsolutions B.V., Delft) as described in de Groot et al. [17]. Alternatively, vaccination was performed via intramuscular injection or subcutaneous injection, as indicated. Two weeks after each immunization, blood samples were collected from the lateral tail vein. Blood was left to clot at 37 °C for 30 min, and serum was collected by taking the supernatant from two consecutive centrifugations at 14,000 RPM. For the second experiment, three weeks after the final immunization, the mice were sedated with isoflurane and challenged intranasally with $2xLD_{50}$ of mouse-adapted pH1N1 virus. The bodyweight of infected mice was monitored for 11 days starting from the day of challenge. Mice that lost 25% or more of their bodyweight relative to the day of challenge, were euthanized by cervical dislocation.

5.7. Hemagglutination inhibition assay

HAI activity was tested, as previously described. Briefly, sera were incubated at 56 °C for 10 min to inactivate complement factors. Each volume of serum was treated with four volumes of receptor destroying enzyme (cholera filtrate from *Vibrio cholerae* culture fluid, Sigma-Aldrich) to remove non-specific agglutinins. Two-fold serial dilutions of treated serum samples were mixed with four hemagglutination units of

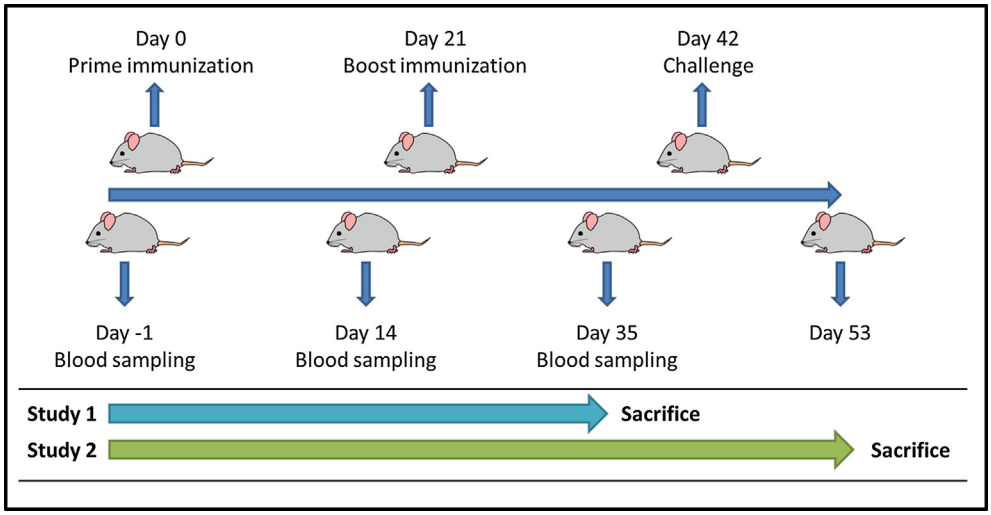

Fig. 6. Immunization, sampling and challenge scheme of the two animal studies.

pH1N1 virus in a final volume of 50 µl and were incubated for 1 h at room temperature. Equal volumes of a 0.5% chicken red blood cell suspension were added and HAI titers were recorded 30 min later. Endpoint titers were defined as the dilutions at which hemagglutination was inhibited completely. A value corresponding to 50% of the limit of detection was assigned to each sample for which no binding was detected.

5.8. Virus neutralization assay

For the virus neutralization assay the sera were incubated for 10 min at 56 °C to inactivate complement factors and were treated with receptor destroying enzyme. Three-fold serial dilutions of serum prepared in OPTIMEM medium (Thermo Fisher Scientific, Massachusetts) were mixed with 50 plaque forming units (PFU) pH1N1 and incubated at 37 °C for 1 h. Subsequently fifty microliters of these samples were added to MDCK cells grown in 96 well plates. After 2 h of incubation at 37 °C 50 µl OPTIMEM medium containing 1.2% Avicel® and 2 µg/ml TPCK -treated trypsin (Sigma-Aldrich, Saint-Louis, MO) was added and the cells were incubated for an extra 42 h. After removing the growth medium, the cells were washed twice with PBS and fixed with 1% paraformaldehyde (PFA), washed 3 times with PBS, permeabilized with 0.1% Triton-X100 (Bio-Rad, Hercules, California), blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis) and subsequently stained with goat anti-RNP influenza A immune serum (BEI Resources, NR-3132). Antigen-specific antibodies were detected with peroxidase-labeled antibodies directed against mouse IgG (Abcam, ab97110). After washing, the plates were incubated for 5 min with TMB substrate. The reaction was stopped by adding an equal volume of 1 M H_2SO_4, and the absorbance at 450 nm was measured. The neutralization EC_{50} values were calculated using non-linear regression analysis in GraphPad Prism (v6.07) software for Windows (GraphPad Software, San Diego, CA). A value corresponding to 50% of the limit of detection was assigned to each sample for which no binding was detected.

5.9. Statistical analysis

For statistical analysis GraphPad Prism was used. Differences in antibody titers, were tested by using a 1-way ANOVA with a Tukey's multiple comparisons test for non-normally distributed data points and a Dunn's multiple comparisons test for normally distributed data points.

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Competing financial interests

B.S. and X.S. declare no competing financial interest. P.J. Vos is CEO of MyLife Technologies. KvdM is co-owner of uPRAX microsolutions.

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