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Influenza nucleoprotein DNA vaccination by a skin targeted, dry coated, densely packed microprojection array (Nanopatch) induces potent antibody and CD8$^+$ T cell responses

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Abstract

DNA vaccines have many advantages such as thermostability and the ease and rapidity of manufacture; for example, in an influenza pandemic situation where rapid production of vaccine is essential. However, immunogenicity of DNA vaccines was shown to be poor in humans unless large doses of DNA are used. If a highly efficacious DNA vaccine delivery system could be identified, then DNA vaccines have the potential to displace protein vaccines. In this study, we show in a C57BL/6 mouse model, that the Nanopatch, a microprojection array of high density (>21,000 projections/cm^2), could be used to deliver influenza nucleoprotein DNA vaccine to skin, to generate enhanced antigen specific antibody and $CD8^+$ T cell responses compared to the conventional intramuscular (IM) delivery by the needle and syringe. Antigen specific antibody was measured using ELISA assays of mice vaccinated with a DNA plasmid containing the nucleoprotein gene of influenza type A/WSN/33 (H1N1). Antigen specific $CD8^+$ T cell responses were measured *ex-vivo* in splenocytes of mice using IFN-γ ELISPOT assays. These results and our previous antibody and $CD4^+$ T cell results using the Nanopatch delivered HSV DNA vaccine indicate that the Nanopatch is an effective delivery system of general utility that could potentially be used in humans to increase the potency of the DNA vaccines.

Keywords: DNA vaccination, Skin vaccination, Needle-free vaccination, Enhanced immunogenicity, Microneedle.

1.0 Introduction

DNA vaccines are inexpensive, easy and faster to produce than protein vaccines [1]. They are stable at room temperature, and could induce strong long lasting humoral and cellular immune responses that confer protection against the disease [2]. Upon delivery of DNA vaccines, plasmids in the vaccines are taken up by host cells, which in turn express the encoded protein antigen [3]. Subsequently, antigen is secreted, or processed and presented by the transfected cell to induce $CD4^+$ T cell, $CD8^+$ T cell, and antibody-mediated immunity. Antigen coding DNA sequences can be rapidly cloned into plasmids using PCR techniques, and could be improved further by gene sequence modifications, or the inclusion of adjuvanting sequences into the plasmid vector [4-6]. Furthermore, multiple antigens can be delivered in a single vaccination to cover a broad range of antigens [7, 8]. Vaccination with DNA vaccine encoding influenza virus nucleoprotein protein (NuPr) induces strong $CD8^+$ T cell responses, which correlate with protection against challenge infection with homologous or heterologous virus in the BALB/c mouse model [9]. Although DNA vaccines are immunogenic and protect against disease in small animal trials, generally DNA vaccines have not been as effective at inducing strong immune responses in humans [10]. Large doses of about 4000 µg DNA vaccine are generally required in human vaccinations; and IM delivery using the needle and syringe is the preferred route of vaccination [11]. A DNA vaccine encoding avian H5 antigen was effective in a prime boost regime 4000 µg of DNA vaccine as the prime and 90 µg of protein vaccine as the boost, to induce responses in 81% of patients, and was more effective than priming with monovalent inactivated H5 protein vaccine [12]. The doses of DNA vaccine used in these studies are very high. Future directions for DNA vaccine translation into humans will require vaccines that are highly effective with lower doses, the use of novel adjuvant strategies, and delivery modalities tailored to enhance immunogenicity.

DNA vaccine delivery to skin have been accomplished in the past using various methods such a tape-stripping, microdermabrasion, ballistic penetration, electroporation and microneedles to breach the skin barrier function [13-24] to allow DNA vaccines to diffuse within the skin into the APCs in the epidermis and dermis. Gene gun has been a popular method of delivering DNA vaccines to skin in the past [25-30]. However gene gun requires complicated and expensive equipment, and the DNA coated on the gold particles are bombarded into skin at very high velocities of around 600 m/s which may damage DNA and also cause extensive cell death. Furthermore, the vaccine dose reductions achieved by delivery methods other than the Nanopatch or microneedles are relatively low.

Three elegant studies done in a mouse model by Y-C Kim et al show that using microneedles, a low dose of 3 µg of haemagglutinin (HA) DNA induced stronger immune response and better protection against influenza virus challenge than 10 µg delivered IM [13, 31, 32]. Furthermore they were able to induce cross protection by co-vaccination of HA DNA with an inactivated whole influenza virus [32]. These studies have been done with microneedles with

a length of 700 µm. In contrast, the length of the microprojections of the Nanopatch is only 110 µm and therefore would not cause much pain or bleeding.

The Nanopatch is a 4x4 mm microprojection array (>21,000 projections/cm^2) **(Fig 1)**, designed using probabilistic calculations to greatly enhance immunogenicity of vaccines by delivering vaccines directly to the immediate vicinity of antigen presenting cells (APCs) which are abundant within the skin [33].

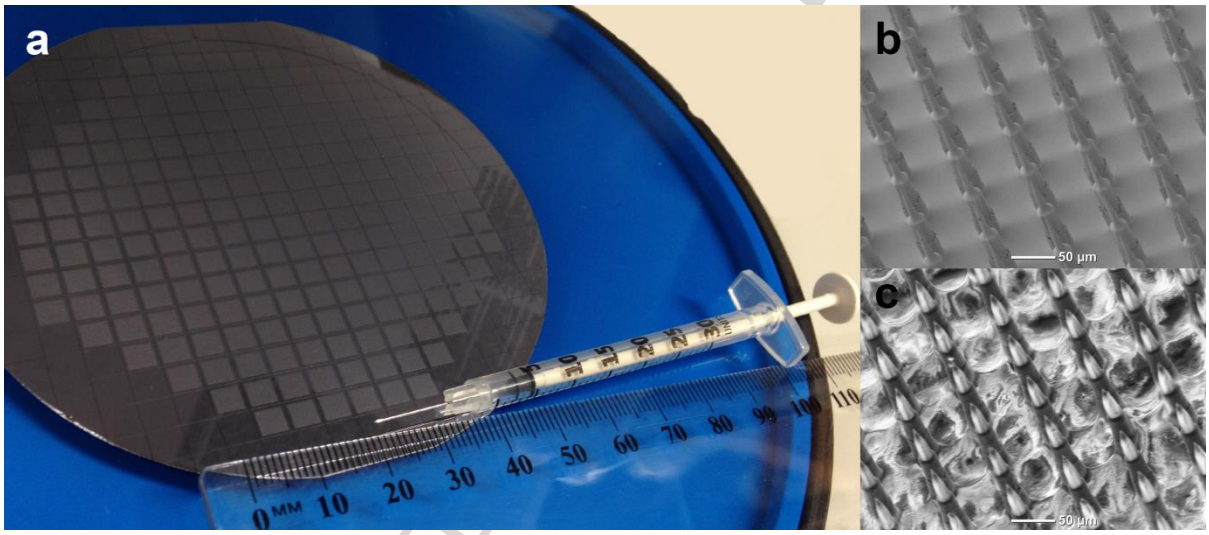

Figure 1 (a) The comparative size of needle and syringe use in influenza vaccinations and a wafer containing multiple Nanopatches. (b) Scanning Electron Microscopy picture of an individual Nanopatch showing the microprojections, and (c) microprojections dry coated with the vaccine.

The vaccines are dry coated onto microprojections, and then dynamic application by a spring loaded applicator is used to drive the microprojections coated with vaccine into the skin, where the vaccine dissolves in the viable epidermis and dermis [34]. Delivery of vaccines by Nanopatch has itself showed a "physical immune enhancer" effect (without the addition of any chemical adjuvants to the vaccine) when delivering vaccines in comparison to conventional delivery routes such as intramuscular delivery using the needle and syringe [35]. Small doses of vaccine delivered by the Nanopatch have led to greatly enhanced immune responses compared to higher IM doses [33]. These responses can be further improved when an adjuvant is included on the Nanopatch [34]. Furthermore, DNA vaccine encoding the extracellular domain of HSV-2 gD2 and delivered by Nanopatch increased seroconversion rates and enhanced antibody titres and $CD4^+$ T cell levels to encoded antigen, compared to conventional IM delivery [22, 23]. These studies show that the Nanopatch has the potential to be used in human vaccinations to enhance efficacy of DNA vaccines, whereas the intramuscular needle/syringe injections have failed to induce significant levels of antibody unless very large doses of vaccine are used.

In the present study, we compared the efficacy of DNA vaccine pVAX1 (Invitrogen, Life Technologies) encoding the Nucleoprotein gene (NuPr) of influenza virus type A/WSN/33 delivered by the Nanopatch against the conventional needle and syringe IM delivery to induce antibody and cellular immune responses, and to determine the general utility of the Nanopatch using a different antigen to that used in our earlier HSV study. Influenza virus NuPr was used as the model antigen in this study because of its highly conserved sequence among the various past and present influenza virus strains circulating amongst human and animal populations. Nanopatch has the potential to further DNA vaccine research in humans; a Nanopatch delivery strategy targeting NuPr antigen could be of high utility in inducing broad immunity against influenza viruses through conserved $CD8^+$ T cell epitopes, as well as specific antibody responses to known and unknown circulating influenza strains. A strategy such as this has greater chance of preventing disease and mortality burden associated with influenza pandemics in humans worldwide. We have previously shown that Nanopatch delivered HSV-2 gD2 DNA vaccine induced high antibody titres and afford protection against HSV-2 challenge in the BALB/c mouse model. The present study was done in the C57BL/6 mouse model to determine whether Nanopatch delivery is of general utility in delivering different DNA vaccines to obtain enhanced immunogenicity.

2.0 Materials and Methods

2.1 Materials

Methylcellulose (MC) (Methocel 60 HG Fluka) was purchased from Sigma-Aldrich (CastleHill, NSW, Australia). ^{14}C labelled ovalbumin was from American Radiolabelled Chemicals (St Louise, MO, USA). Purified Quil-A saponin adjuvant was from Brenntag Biosector, Denmark. β-Galactosidase (β-Gal) protein was from SIGMA, Cat# G5635-3ku. Nucleoprotein (NuPr) gene is from influenza type A/WSN/33 (H1N1) [36] cloned into the plasmid pET28a is a gift from Dr. Yizhi Jane Tao at Rice University, Houston, Texas, USA. Plasmids pVAX1 and pVAX1-β-Galactosidase (pVAX-β-Gal) are from Invitrogen, Life Technologies. The NuPr gene was cloned into pVAX1 and the plasmid (pVAX-NuPr) characterised and purified to remove endotoxins (levels< 10 EU/μg DNA). Recombinant Nucleoprotein was expressed from pET28a plasmid and purified by University of Queensland Protein Expression Facility (PEF), Brisbane, Australia.

2.1.1 Mice

Female C57BL/6 mice aged 6 to 10 weeks old at the start of vaccination were purchased and maintained under standardised specific pathogen free conditions in the animal facility of the Australian Institute of Biotechnology and Nanotechnology (AIBN), University of Queensland, Brisbane, Australia. All animal care and experiments were conducted in accordance with NHMRC (Australia) guidelines and with the approval of the University of Queensland animal ethics committee.

2.2 Methods

2.2.1 Nanopatches

Nanopatches were manufactured according to published method [37]. Each Nanopatch (4×4 mm) was coated with either pVAX-NuPr or pVAX-β-Gal using a nitrogen jet drying coating method [23, 38]. The coating solutions were composed of viscosity enhancer methyl cellulose (10 mg/ml), and pVAX-NuPr or pVAX-β-Gal at 3-5 mg/ml diluted with PBS. The skin delivered doses of pVAX-NuPr or pVAX-β-Gal were derived from total amounts on the Nanopatch corrected for skin delivery percentage calculated from the ^{14}C-Ovalbumin tracer experiments as described [34]. For coating, 8 µl of coating solution was applied onto each Nanopatch and a nitrogen gas jet was applied to controllably localise the vaccine onto the projections as described [38].

2.2.2 Quantification of vaccine delivered to skin by the Nanopatch

C57BL/6 were anesthetised and then patches dry-coated with pVAX-NuPr mixed with tracer amounts (9 µCi) of ^{14}C-Ovalbumin were applied to the inner lobe of the ear, and holding in place for 2 min for the vaccine to diffuse within the epidermis and dermis as described previously [39]. After the application and removal of the Nanopatch, the ear surface was swabbed three times with a cotton-tipped swab moistened with PBS to remove vaccine that is deposited on the skin surface and mice were sacrificed and patched ears excised. Swabbing was done to remove pVAX-NuPr remaining on the surface of the skin and not gone into the skin. Swab tips, excised ears, used Nanopatches, and 8 µl samples of vaccine coating solution were placed in individual scintillation vials. To extract the vaccine containing the tracer protein into aqueous phase from swabs and patches before liquid scintillation counting, 1 ml of PBS was added to vials containing swabs, used Nanopatches, and coating solution and the vials were vortexed thoroughly followed by subsequent incubation overnight at 23°C. A tissue solubiliser (Solvable™ Perkin Elmer) was added to vials containing excised ears (1 ml per vial) to solubilise the ear tissue. Ears were solubilised overnight at 60°C. All samples were subjected to vigorous vortexing before the addition of 10 ml of scintillant (Ultima Gold™, Perkin Elmer). Disintegrations per minute (DPM) were measured for 10 min in a PerkinElmer TriCarb 2810 TR liquid scintillation counter with automated quench correction and colour compensation. The amount of pVAX-NuPr delivered to the skin was determined by the radioactivity present in the ear sample as a percentage of that originally coated on the patch.

2.2.3 Vaccination of mice

C57BL/6 were vaccinated by applying patches dry coated with pVAX-NuPr or pVAX-β-Gal to the inner lobe of each ear (one patch per ear, two patches per mouse), and holding in place for 2 min for the vaccine to diffuse into the epidermis/dermis [39]. In all experiments, a booster dose was given to the same vaccination site 7 days after the first vaccination. PVAX-NuPr DNA vaccine expresses NuPr of influenza virus A/WSN/33 strain under control of the CMV promoter. Antibody responses to pVAX-NuPr were compared to the pVAX-β-Gal

control vector vaccinated mice expressing β-Gal. Additional groups of mice were vaccinated using the needle and syringe in the caudal thigh muscle or intra dermally in ear skin for comparison. All mice were bled 3 weeks after the vaccination. The sera were separated and stored frozen at −20°C until assays were performed.

2.2.4 Vaccine antigen specific antibody analyses by Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were performed as previously described [40]. Briefly, the flat bottomed 96 well ELISA plates (Nunc Maxisorp, Denmark) were coated with Nucleoprotein protein antigen at a concentration of 50 µg/ml in 0.1 M sodium bicarbonate buffer and 50 µl of this solution was added to each well and incubated overnight at 4°C. The plates were blocked with 4 mg/ml BSA in PBS and were used to determine the titres of antigen specific IgG induced. The sera were diluted in doubling dilutions starting from 1:100 up to 1:204,800 before transferring onto ELISA plates. Bound antibodies were detected by the addition of horseradish peroxidise conjugated goat anti-mouse IgG. The colour development was performed using ABTS (2,29-azino-bis3-ethylbenzthiazoline-6-sulfonic acid; Sigma Cat# A-1888) as the substrate. The absorbance readings at 405 nm were measured against control wells containing no serum.

2.2.5 $CD8^+$ T Cell Epitope Peptide

NP366 synthetic peptide containing the H-$2D^b$ restricted CD8 T cell epitope 366-374 (Amino Acid Sequence: ASNENMETM) [41, 42] was chemically synthesised to >90% purity by Auspep Australia and used in the ELISPOT assays.

2.2.6 Quantification of $CD8^+$ T cell response (IFN-γ ELISPOT)

Vaccinated mice were euthanised by cervical dislocation and spleens were harvested on day 14 or 21 after the vaccination (for single vaccination or double vaccination respectively). IFN-gamma Enzyme-Linked Immunospot (IFN-γ ELISPOT) assays were performed to quantify antigen specific $CD8^+$ T cells using a conserved H-$2D^b$ restricted epitope peptide (NP366) of NuPr. This procedure was based on a publication with slight modification [43]. Briefly, spleens were disrupted to obtain splenocytes. 96 wells ELISPOT plates (Millipore MultiScreen-HA Cat#MAHAS4510) were coated with IFN-γ capture antibody (MabTech Cat# 3321-3; final concentration of 8 µg/mL). Splenocytes and a $CD8^+$ T cells epitope specific peptide NP366 at 1 µg/ml), was used to stimulate cells *in vitro* at 37°C in 5% CO_2 for 40 to 44 hours. An anti-IFN-γ biotinylated detection antibody (MabTech Cat# 3321-6; final concentration of 1 µg/ml) was added and incubated for 2 hours in room temperature. Avidin horseradish peroxidase (HRP) (Sigma Cat# A-3151) was added and the plate was incubated for 1 hour in room temperature. The substrate, DAB (Sigma Cat# D0426) was prepared according to the instructions in the package, was added to the wells and kept until spots appear (1 to 5 minutes). The spots were counted using AID ELISPOT reader System (© AID

Autoimmun Diagnostika GmbH, 2003). The number of spots per added total number of splenocytes to each well reported has been background subtracted by their respective negative control (without peptide).

2.2.7 Statistical analysis

All data were analysed via 2-tailed unpaired Student's t test using GraphPad Prism version 6.05 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All data represented are expressed as mean ± standard error of mean (SEM). A difference was considered statistically significant using Student's t-test when $p < 0.05$.

3.0 Results and Discussion

3.1 Quantifying the percentage of vaccine delivered to the skin by the Nanopatch

The Nanopatch delivers dry coated vaccine via an array of microprojections which penetrate the skin to deliver the vaccine payload into the epidermis and dermis. Vaccine dissolves from the projections within the skin upon rehydration. Not all the vaccine coated on the patch is delivered into skin. To accurately determine a delivered dose, we used an established technique using radioactive ^{14}C-ovalbumin, which was included in the vaccine solution to act as a tracer upon dissolution of the dry coated vaccine payload within the skin [23]. Using this technique, we determined that 37.5% of the dry coated DNA vaccine dissolved off the patch and was delivered into the skin, while 60% remained on the Nanopatch and 2.5% was detected in the swab which is the amount that is deposited on the surface of the skin (**Fig 2**). These results are consistent with our previous findings [23]. The delivered dose is defined as the amount of antigen that is delivered into the skin by the Nanopatch. ^{14}C counts in ear skin/(^{14}C counts on skin + swab + patch)] × 100% = [5398/(5398 + 82 + 8864)] × 100% = 37.5% ± 3% (mean ± SEM, N = 10). The delivery efficiency was determined to be 37.5%. This value was used to calculate the delivered dose of vaccine throughout this study.

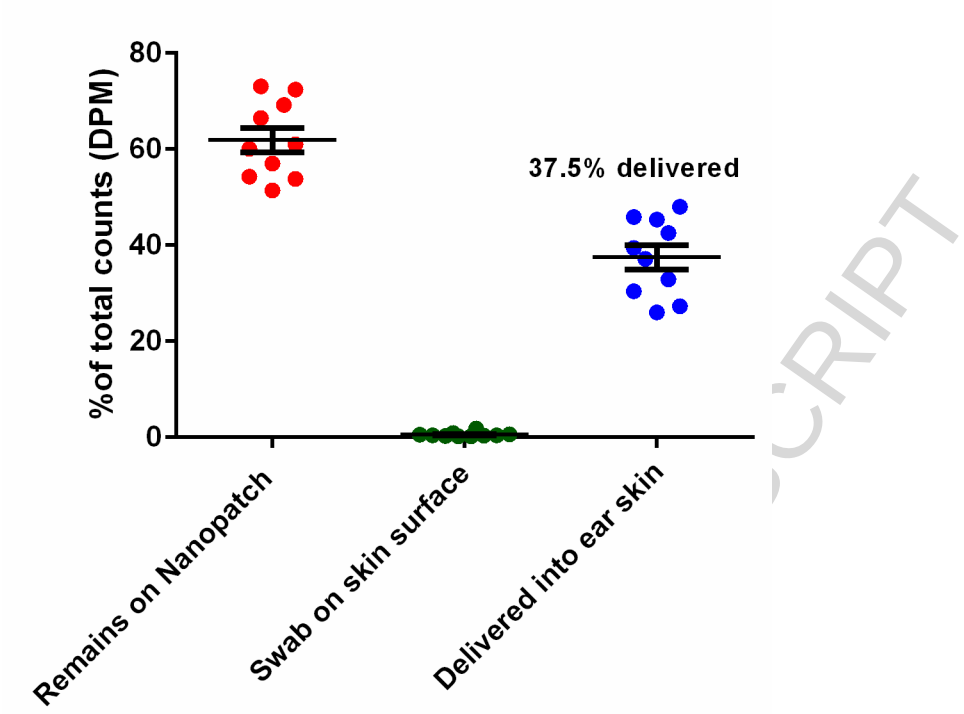

Figure 2: Determining the percentage of vaccine delivered to skin using ^{14}C radioactive tracer. The percentage of vaccine that is transferred from the Nanopatch to the ear skin was determined using ^{14}C labelled ovalbumin as the tracer. The amount of vaccine formulation delivered to the ear skin, left behind on the skin surface (swab), and remaining on the Nanopatch were quantified by measuring the radioactivity using a liquid scintillation counter. The skin delivered vaccine dose for the Nanopatch was calculated by this method and used in all experiments.

3.2 pVAX-NuPr DNA vaccine delivered by the Nanopatch induced enhanced antibody levels compared to conventional IM delivery.

We compared the immunogenicity of Nanopatch delivered pVAX-NuPr to that delivered by the classical IM route which is the most common route of DNA vaccine administration. Mice were vaccinated with 10 µg or 50 µg of pVAX-NuPr delivered IM, or with 1 µg or 10 µg delivered by the Nanopatch, the similar patch doses we used in our earlier DNA vaccine study [22]. Mice were vaccinated at day 0 and given a booster vaccination at day 7 and were bled and sera were collected at day 21 and analysed for NuPr-specific antibody by ELISA (**Fig 3**). Antibody titres induced by the delivery of 1 µg of pVAX-NuPr by Nanopatch were comparable, and not different from the antibody titres induced by 10 µg and 50 µg of IM delivered vaccine indicating greater than 10 fold dose reductions could be achieved using the Nanopatch delivery. Furthermore, 10 µg of pVAX-NuPr delivered by Nanopatch induced a significantly greater NuPr-specific antibody response than 10 µg delivered IM, or 50 µg (***p<0.05*). These data show a dose sparing effect when using the Nanopatch, where 1 µg of DNA vaccine, 1/10th of a conventional IM dose of 10 µg, generated comparable antibody titres, and 10 µg of DNA vaccine delivered by the Nanopatch afforded a 1.2 log-fold increase of mean values in NuPr-specific antibody response compared to classical IM delivery. Enhanced antibody response to a DNA vaccine using a different antigen delivered by the

Nanopatch has also been previously reported by us [22]. Our previous study using an HSV plasmid in BALB/c mice shows very similar antibody pattern with 1 µg vaccine delivered by the Nanopatch to induce a similar response to 10 µg delivered IM and 10 µg delivered by the Nanopatch to be superior to 10 µg delivered IM [22]. These two studies and a microneedle study [13] combine to reinforce the hypothesis that Nanopatch or microneedle delivery is effective in enhancing the immunogenicity of DNA vaccine-encoded antigens to the skin where a high concentration of immunologically active cells reside. Furthermore, microprojections of the Nanopatch is generating "physical immune enhancer" effect by controlled cell death releasing endogenous danger signals that will help enhanced immunogenicity to vaccines released in the immediate vicinity [35].

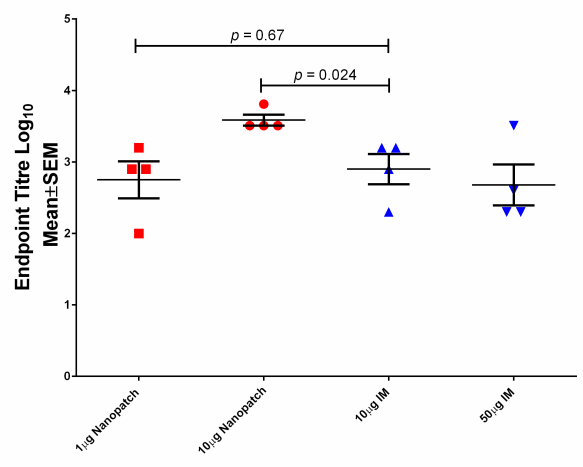

Figure 3: Antibody response titre from mouse sera 21 days post vaccination. DNA vaccine encoding pVAX-NuPr was used in the vaccinations with the Nanopatch or with intramuscular (IM) vaccination route. 1 µg or 10 µg vaccine doses were delivered to skin using the Nanopatch while 10 µg or 50 µg were delivered to caudal muscle using the needle and syringe. Mice were vaccinated at day 0 and given a booster vaccination at day 7 and bled on day 21. Sera were analysed by ELISA.

3.3 Nanopatch delivered pVAX-NuPr DNA vaccine induces an enhanced $CD8^+$ T-cell response.

It is known that $CD8^+$ T cell mediated immunity plays a critical role alongside $CD4^+$ T cells in immunity against influenza virus [44, 45]. Therefore we next determined whether this DNA vaccine is also capable of inducing an enhanced $CD8^+$ T cell response in addition to the enhanced antibody response, when delivered to the skin by the Nanopatch. We selected a sequence conserved H-$2D^b$ restricted epitope peptide (Sequence: ASNENMETM) of NuPr and using IFN-γ ELISPOT assays to measure the $CD8^+$ T cell response generated. Mice were vaccinated with pVAX-NuPr (or as a control with pVAX-β-Gal) using the Nanopatch, and 15 days later splenocytes were harvested and cultured overnight with or without NuPr epitope peptide and spots counted (**Fig 4A**). There was a significant number of splenocytes from mice vaccinated with Nanopatch delivered pVAX-NuPr that secreted IFN-γ specifically after incubation with NuPr peptide, when compared to splenocytes from mice vaccinated with the irrelevant control pVAX-β-Gal (**Fig 4A**). These data show that a $CD8^+$ T cell mediated

immune response to a conserved NuPr epitope was induced by pVAX-NuPr vaccine delivered by the Nanopatch and it is specific for the NuPr. We then compared the number of IFN-γ secreting splenocytes from mice vaccinated with different doses of pVAX-NuPr delivered IM or by Nanopatch to determine if there an enhancement of $CD8^+$ T cell immunogenicity when the vaccine is delivered by the Nanopatch compared to the IM injection. Spots generated from unstimulated splenocytes were subtracted from the number of spots from peptide stimulated splenocytes to determine NuPr-specific IFN-γ secreting cells. There was a significantly greater number of IFN-γ secreting splenocytes from mice vaccinated with 10 µg of pVAX-NuPr delivered by Nanopatch, compared to all doses of pVAX-NuPr delivered IM (**Fig 4B**). In mice, DNA vaccine-mediated cellular immunity to matrix and NuPr has been shown to protect against lethal influenza challenge [46, 47], and correlated with IFN-γ secreting splenocytes. A study using pVAX-NuPr as the DNA vaccine with HMGB1 as an adjuvant delivered by the IM injection with electroporation treatment immediately after, did show enhanced protection, cellular immune response and survival in lethal influenza virus challenge [47]. However the disadvantage of this method is the use of electroporation which needs electricity and specialised equipment which is difficult to be used in mass vaccination programmes especially in resource poor countries. In contrast, Nanopatch vaccine delivery using this simple device provides enhanced antibody as well as an enhanced $CD8^+$ T cell mediated immune response [34, 48], is ideal to provide protection against influenza viruses [49].

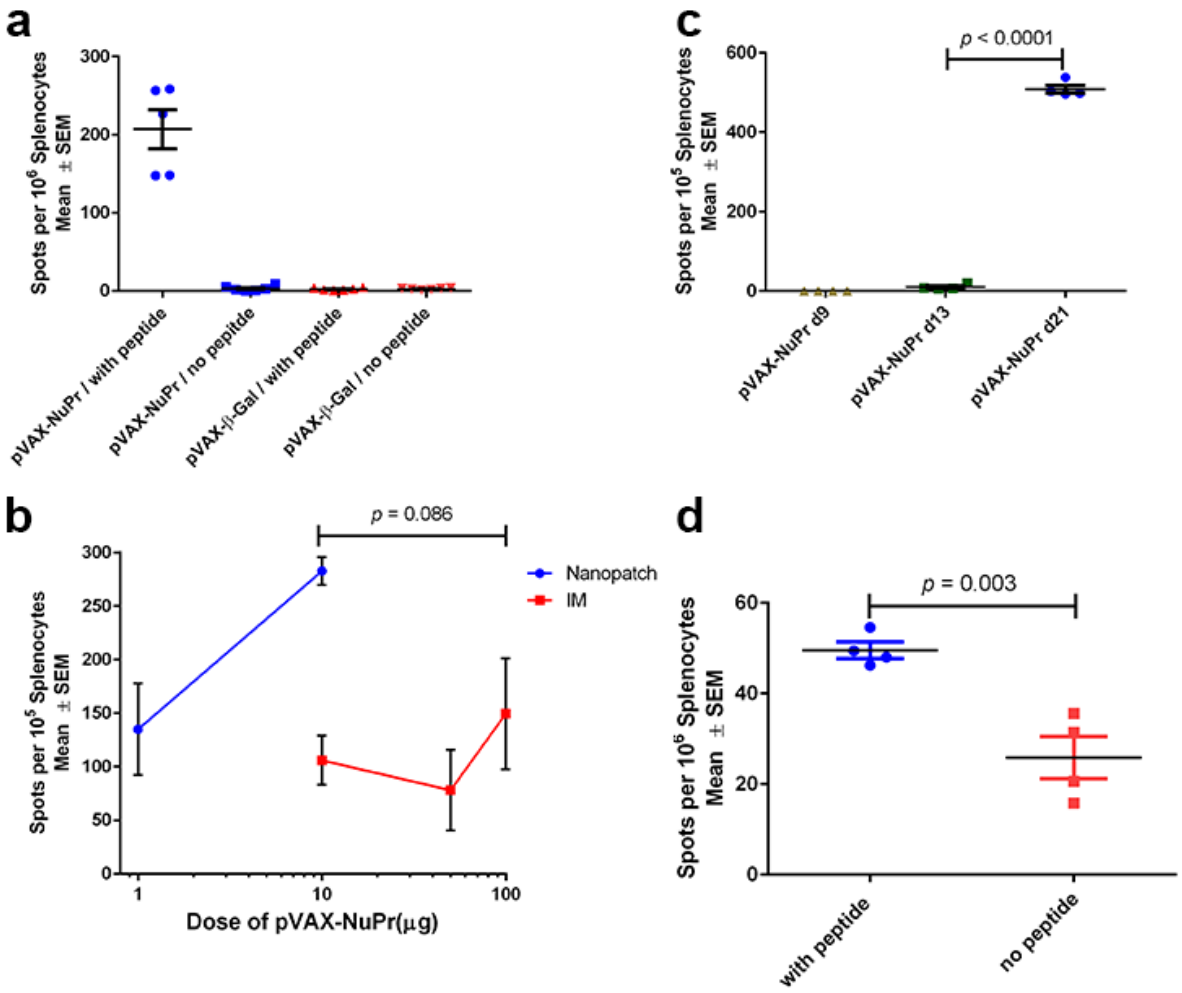

Figure 4: IFN-γ ELISPOT assay results showing NuPr-specific CD8$^+$ T cell activity induced in response to the pVAX-NuPr DNA plasmid vaccinations. (a) Detection of NuPr-specific IFN-γ secreting CD8$^+$ T cells using the epitope specific ASNENMETM peptide. Splenocytes from mice vaccinated and boosted (day 0 and 7) with 30 µg of DNA plasmid vaccine pVAX-NuPr or as a control with pVAX-β-Gal and spleens harvested at day 15 post vaccination and incubated with or without the CD8$^+$ T cell epitope peptide. **(b) IFN-γ secreting CD8$^+$ T cell response induced by Nanopatch compared to IM**. Responses from mice vaccinated at day 0 and boosted at day 7 by the Nanopatch or intramuscular (IM) injection with various doses of DNA plasmid vaccine pVAX-NuPr and spleens harvested at day 21 post vaccination. **(c) Kinetics of antigen specific IFN-γ secreting CD8$^+$ T cell response generated post Nanopatch vaccination.** Mice were vaccinated at day 0 and boosted at day 7. Splenocytes were harvested at 9, 13, 21 days post vaccination and day 21 shown a significant increase of antigen specific IFN-γ secreting CD8$^+$ T cell response *(***p<0.001*). **(d) Long lasting antigen specific IFN-γ producing CD8$^+$ T cell response induced by Nanopatch vaccination.** Mice were vaccinated at day 0 and boosted at day 7 with 30 µg of pVAX-NuPr by Nanopatch. Splenocytes were harvested 180 days post vaccination. Long lasting antigen specific IFN-γ producing CD8$^+$ T cell response was detected *(**p<0.01*).

3.4 Kinetics of antigen specific $CD8^+$ T cell generation in response to Nanopatch delivered pVAX-NuPr DNA vaccine

We then determined the minimum time interval that would take to generate a detectable antigen specific $CD8^+$ T cell response in spleen after Nanopatch skin delivery of the pVAX-NuPr DNA vaccine. Groups of mice were vaccinated with 10 µg of pVAX-NuPr, spleens were harvested at different days post vaccination (9, 13 and 21), and cultured *ex vivo* with/without NuPr $CD8^+$ T cell epitope peptide in an ELISPOT assay (**Fig 4C**). A significant number of IFN-γ secreting splenocytes were not detectable until day 21, at which time there was a peak of 508±20 $CD8^+$ T cell epitope-specific IFN-γ secreting cells (****p<0.001*). These data show that NuPr-specific $CD8^+$ T cell mediated responses are not detectable up to day 13, after which significant expansion of T cells (~50-fold) occurs by 21 post vaccination. The lag time of about one week compared to direct subunit protein vaccination may be due to the fact that DNA vaccine has to generate the protein first and the protein then will induce the antibody. Conventional intramuscular vaccination schedule with DNA vaccines for optimal $CD8^+$ T cell activity is day 0, 14, 28 and harvesting the splenocytes at day 35 to 42 [47, 50, 51]. These studies did not report the kinetics of T cell generations with time.

3.5 Nanopatch delivered pVAX-NuPr DNA vaccine induce long lasting $CD8^+$ T cell responses

We then determined whether DNA plasmid vaccination using the Nanopatch delivery induces a long lasting $CD8^+$ T cell response to the NuPr. Long lasting responses are required for a successful vaccination disease protection. Mice were vaccinated and boosted once after 7 days with 30 µg of pVAX-NuPr, and left for 180 days. Then splenocytes were harvested and cultured in an ELISPOT assay with or without NuPr $CD8^+$ T cell epitope peptide, and IFN-γ secreting splenocytes were quantified (**Fig 4D**). There was a significantly number of nucleoprotein specific IFN-γ secreting $CD8^+$ T cells in splenocytes from mice vaccinated with pVAX-NuPr delivered by Nanopatch, when stimulated with peptide, compared to unstimulated splenocytes. These data show that a long lasting influenza NuPr specific $CD8^+$ T cell response could be generated when the DNA vaccine is delivered by the Nanopatch (***p<0.01)*. Combined, these data indicate that the $CD8^+$ T cell mediated immune response induced by Nanopatch delivered pVAX-NuPr DNA vaccine detectable at 21 days *post* vaccination and persists for up to at least 180 days *post* immunisation. A high percentage of the latter are considered to be the stable memory T cell pool, which can persist for over 500 days [52]. In humans, immune responses to highly conserved NuPr and matrix proteins are immunodominant, and cellular immune responses to these antigens play a critical role in protection against influenza virus infection associated illness [53], and are likely to confer a long term protection against influenza virus associated disease.

4.0 Conclusions

This study shows that the influenza NuPr DNA vaccine delivered to skin using the Nanopatch induced higher levels of NuPr-specific antibody and also $CD8^+$ T cell responses, at lower

doses of vaccine, compared to the conventional IM needle and syringe delivery method. Even though DNA vaccines have several advantages over typical formulated protein vaccines such as thermostability, ease and rapidity of manufacture, the general acceptance of DNA vaccines have been low due to large doses of vaccine required in humans when conventional needle and syringe IM route of delivery is used. Therefore Nanopatch/DNA vaccine delivery system has the potential to be used in influenza pandemics where a vaccine needs to be manufactured rapidly, distributed and vaccinated in a setting with few resources such as refrigeration and need for medically trained personnel. Nanopatch/DNA system will be useful in controlling other disease pandemics as well because of its potential to be mailed to people's homes to be self-administered.

5.0 Acknowledgements

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6.0 Authors Contributions

G.J.P.F. conceived and designed the experiments while M.A.F.K. provided scientific oversight. M.A.F.K. contributed reagents/materials/analysis tools. H.I.N prepared Figures 1 and 2 and J.Z. prepared Figures 3, 4 and Supplementary Figure. S.R.Y. coated all the Nanopatches used in the experiments. Mainly J.Z., with the help of H.I.N and S.R.Y. performed the experiments. G.J.P.F., J.Z., and H.I.N analysed the data. G.J.P.F. and O.L.H. wrote the manuscript, and H.I.N., and M.A.F.K. edited the manuscript.

7.0 Competing financial interests

M.A.F.K. had a financial interest in the commercialisation of the Nanopatch technology. This included him performing roles in Vaxxas Pty Ltd (Founder, Chief Technology Officer and a Director). M.A.F.K and G.J.P.F are inventors on Nanopatch patents licenced to Vaxxas.
G.J.P.F. was employed by Vaxxas Pty Ltd. as the Senior Vaccinologist and H.I.N. was employed by Vaxxas Pty Ltd. as a Coating and Formulation Scientist.

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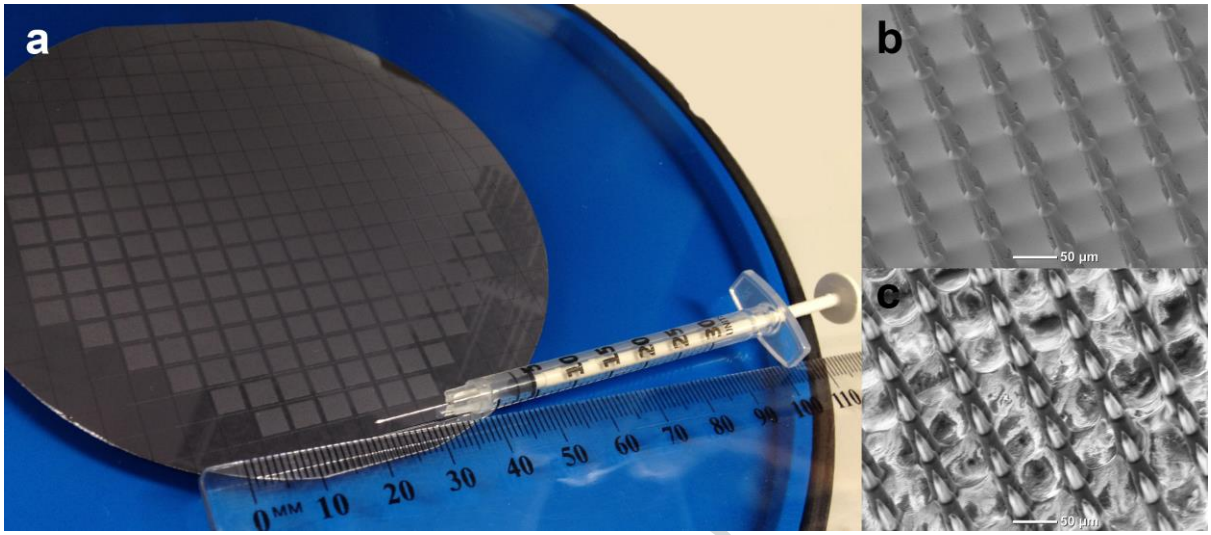


Figure 1

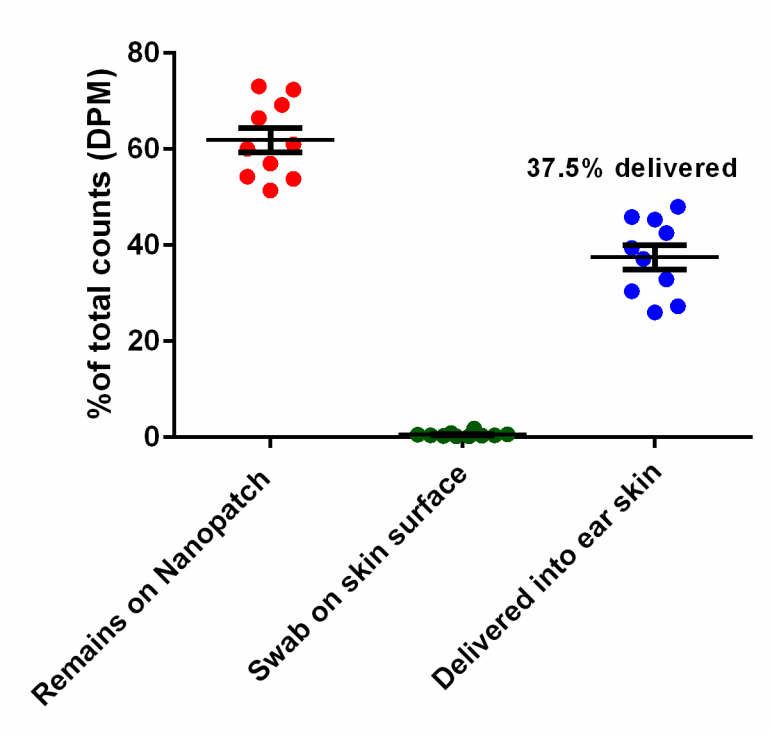


Figure 2

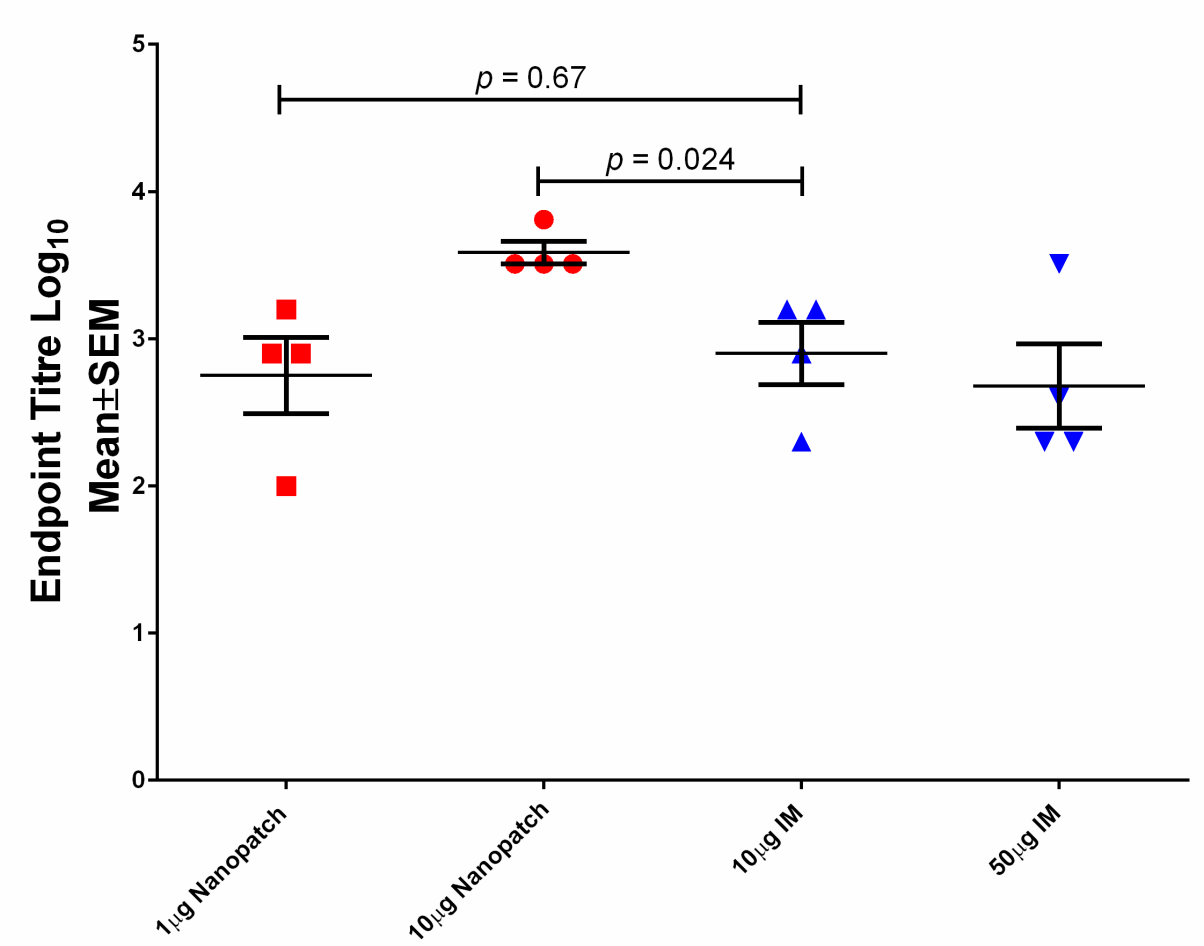


Figure 3

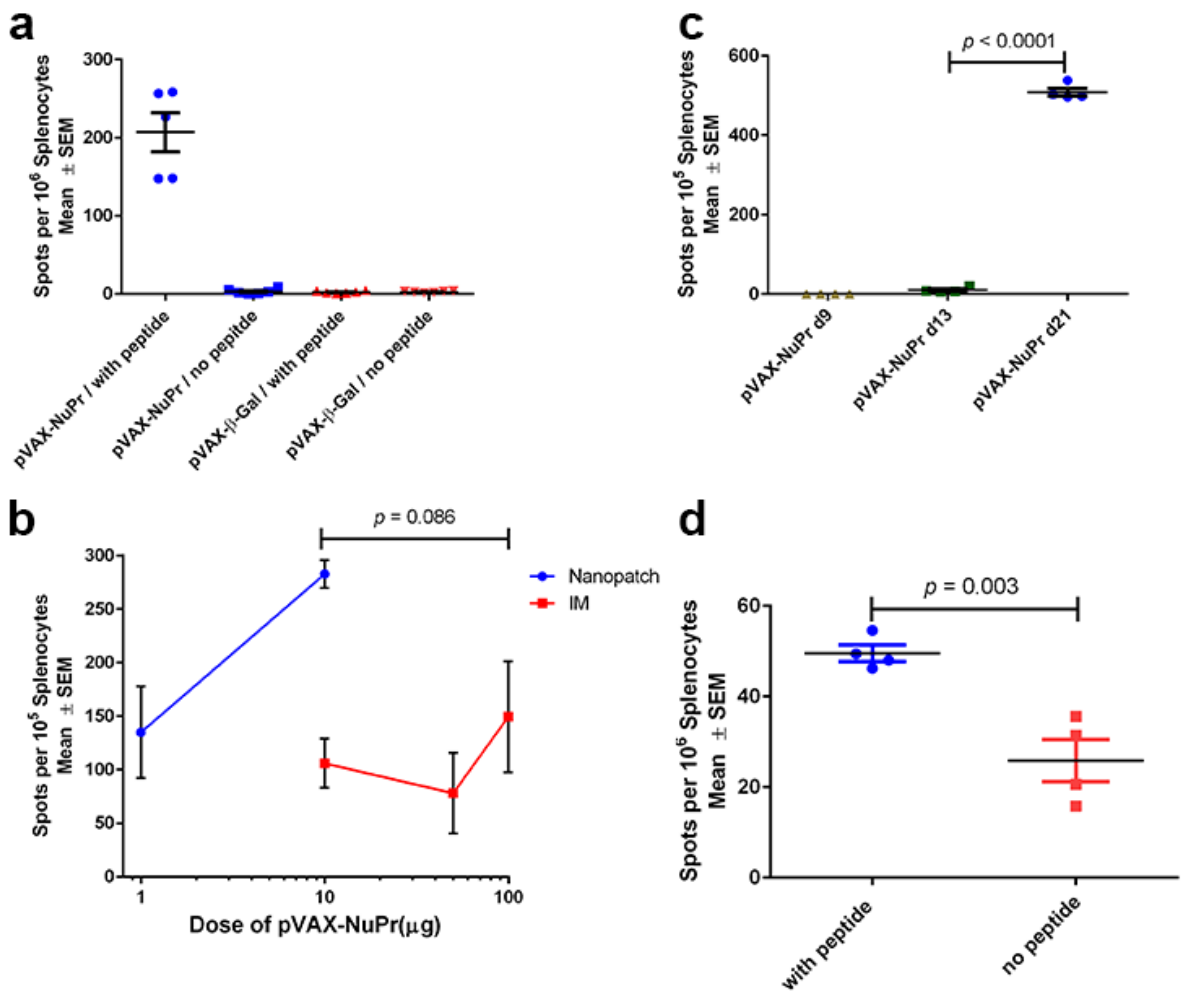
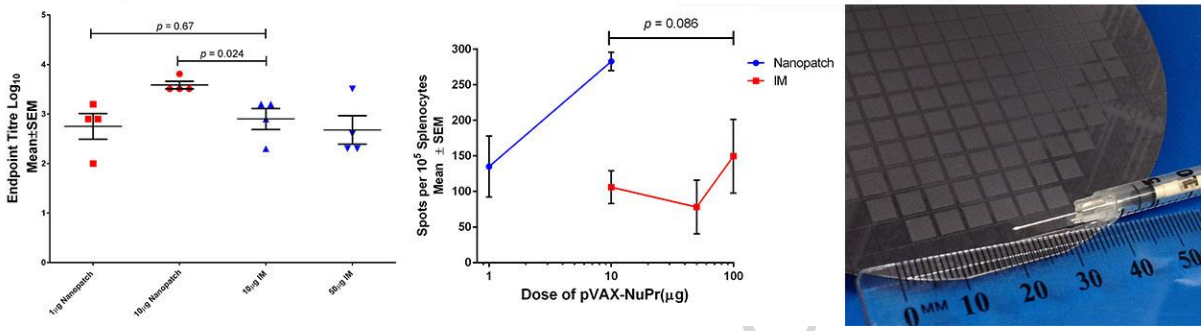


Figure 4



Graphical abstract