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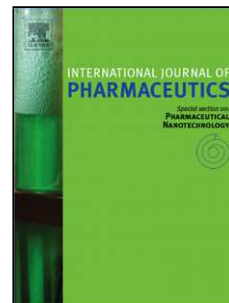
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Skin vaccination using microneedles coated with a plasmid DNA cocktail encoding nucleosomal histones of *Leishmania spp.*

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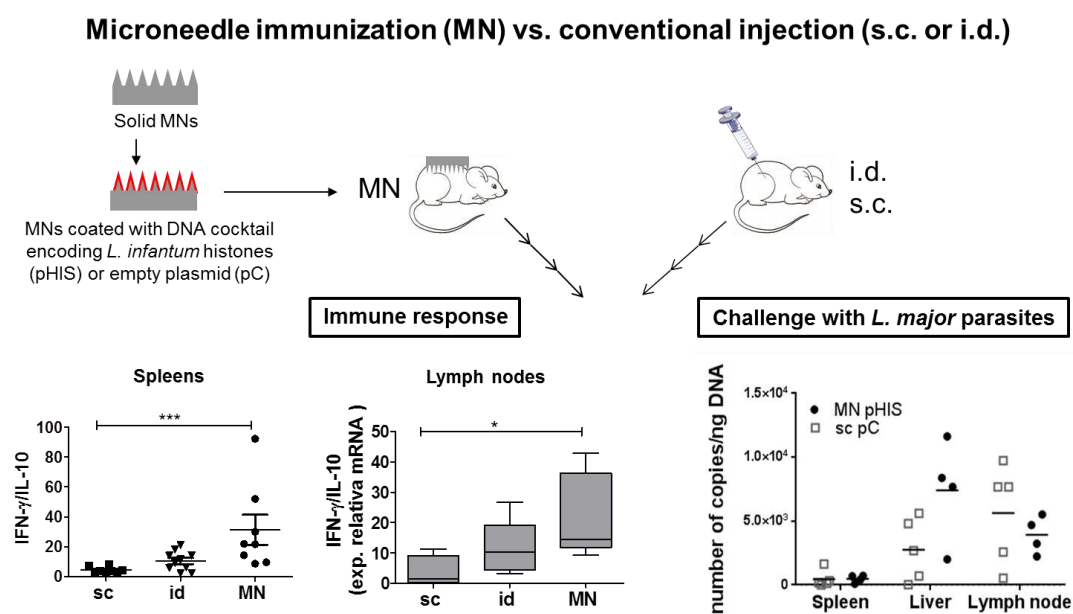
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Graphical abstract



Abstract: Vaccine delivery using microneedles (MNs) represents a safe, easily disposable and painless alternative to traditional needle immunizations. The MN delivery of DNA vaccines to the dermis may result in a superior immune response and/or an equivalent immune response at a lower vaccine dose (dose-sparing). This could be of special interest for immunization programs against neglected tropical diseases such as leishmaniasis. In this work, we loaded a MN device with 60 µg of a plasmid DNA cocktail encoding the *Leishmania infantum* nucleosomal histones H2A, H2B, H3 and H4 and compared its immunogenicity and protective capacity against conventional s.c. or i.d. injection of the plasmid. Mice immunized with MNs showed increased ratios of IFN-γ/IL-10, IFN-γ/IL-13, IFN-γ/IL-4, and IFN-γ/TGF-β in the spleens and lymph nodes compared with mice immunized by s.c. and i.d. routes. Furthermore, CXCL9, CXCL10 and CCL2 levels were also higher. These data suggest that the nucleic acid immunization using MNs produced a better bias towards a Th1 response. However, none of the immunizations strategies were able to control *Leishmania major* infection in BALB/c mice, as illustrated by an increase in lesion size and parasite burden.

Keywords: Microneedle, skin vaccine delivery, DNA vaccine, histone, *Leishmania*

1. Introduction

Leishmaniasis is an infectious disease caused by protozoan parasite species of the genus *Leishmania*. This disease is one of the six major tropical diseases classified by the World Health Organization. Approximately, one billion people are at risk of infection worldwide and more than 1.3 million new infections occur each year (Alvar et al., 2012). Depending on the species, *Leishmania* can cause visceral leishmaniasis (VL), which is fatal if untreated, or cutaneous leishmaniasis (CL). Current treatments are expensive, toxic and do not prevent disease relapse. Drug resistance is also increasing (Akbari et al., 2017). However, people previously infected with *Leishmania* can gain resistive immunity to reinfection and therefore the development and delivery of anti-leishmanial vaccines could be an effective means of controlling or eliminating CL

or VL (Gillespie et al., 2016). Although great efforts have been made in this area, there is still no effective vaccine against human leishmaniasis (Costa et al., 2011).

In recent decades, potential candidate antigens such as killed and lived attenuated parasites, crude parasites, pure or recombinant *Leishmania spp.* proteins, DNA encoding leishmanial proteins or immunomodulators from sand fly saliva have been tested in animal models, but very few candidate vaccines have progressed beyond the experimental stage (Badiee et al., 2013; Jain and Jain, 2015; Khamesipour et al., 2006; Srivastava et al., 2016). DNA vaccines have numerous advantages over other vaccine strategies: (i) simple and cheap to produce, (ii) enhanced temperature stability, (iii) concurrent expression of multiple proteins *in vivo*, which are folded and modified in a comparable manner to their corresponding native proteins, and (iv) ability to elicit Th1 responses and induce both CD4⁺ and CD8⁺ T cells, which is necessary for parasite eradication (Cui, 2005; Kumar and Samant, 2016). A variety of antigens have been studied as DNA vaccine expression products against *Leishmania spp.* including gp63 (Mazumder et al., 2011), LACK (Ramos et al., 2008), CP-Ldcccys1 (Ferreira et al., 2008), ORF (Sukumaran et al., 2003), KMP-11 (Guha et al., 2013), LiPABPs (Soto et al., 2015), LiP0 (Pereira et al., 2015) and LEISHDNAVAX (Das et al., 2014). Whilst varying degrees of efficacy have been demonstrated in animal models, the level of protection elicited by DNA vaccines improves when multiple antigens are co-administered.

There are five main classes of histones and four of them, H2A, H2B, H3 and H4, form the nucleosomal unit core of chromatin. Several studies suggest that *Leishmania spp.* histones are immunologically important during leishmaniasis (Baharia et al., 2014; Chenik et al., 2006; de Carvalho et al., 2003), making them attractive targets in the development of *Leishmania spp.* vaccines. Moreover, it is also known that *Leishmania spp.* histones are highly conserved between different species (Iborra et al., 2004). Studies carried out with these histones of *L. infantum* concluded that the combination of the four nucleosomal histones, expressed via a DNA vaccine, provided stronger immunity than separate or paired histone immunizations. This study also revealed that the DNA cocktail maintained protection against *L. major* reinfection (Carrion et al., 2008b). This plasmid DNA cocktail resulted in a specific Th1 response with enhanced IFN- γ production and low IL-4 levels, which contributed to control *L. major* infection in immunized mice (Iborra et al., 2004). Carneiro et al. also determined that BALB/c mice

immunized with the plasmid DNA mixture expressing four different *L. infantum chagasi* nucleosomal histones (homologous immunization, DNA/DNA) was as effective as the combination of the DNA cocktail followed by the corresponding recombinant proteins and CpG as an adjuvant (heterologous vaccination, DNA/rprotein+CpG) (Carneiro et al., 2012). Furthermore, HISA70, a DNA vaccine composed of the *Leishmania spp.* histones (H2A, H2B, H3 and H4), A2 and HSP70, was able to shift the response away from the more undesirable Th2 pathway, producing high levels of IL-17 and IFN- γ in vaccinated mice. These observations correlated with a complete absence of parasites in spleens after *L. major* infection (Dominguez-Bernal et al., 2012).

DNA vaccines provide promising candidates in the pursuit of new vaccines against *Leishmania spp.* In most cases these vaccines have been administered by the intramuscular (i.m.) or subcutaneous (s.c.) route. However, intradermal (i.d.) vaccination may provide an opportunity to increase the immunogenicity of DNA vaccines (Engelke et al., 2015). Muscle and subcutaneous tissue contains relatively few dendritic cells (DCs) compared to the dermis and epidermis of skin, which are densely populated by different subsets of DCs. These competent antigen presenting cells (APCs) play a central role in developing adaptive immune responses (Banchereau and Steinman, 1998), undoubtedly important in *Leishmania spp.* infection control. The delivery of DNA vaccines to the dermis may therefore result in a superior immune response and/or an equivalent immune response at a lower vaccine dose (dose-sparing) (Kenney et al., 2004; Van Damme et al., 2009). Dose-sparing and the resulting reduction in unit costs could be of special interest for immunization programs in resource-poor areas.

Currently, the Mantoux method is the conventional technique for i.d. vaccination. This technique, used for tuberculosis and rabies immunization, involves insertion of a hypodermic needle into the skin at an acute angle. It is painful and administration requires trained healthcare personnel (Scheiblhofer et al., 2013). Furthermore, the reuse of contaminated needles is a risk, particularly where their supply is short. The introduction of minimally-invasive microneedle (MN) devices may confer a number of advantages for i.d. injection: (i) ease of use and disposal, (ii) painless administration, (iii) reduced risk of infection through minimal blood contact, and (iv) potential for self-administration by patients (Larraneta et al., 2016). In addition, simultaneous delivery of antigen at multiple skin sites using MN arrays could result in effective

distribution of vaccines, leading to superior immune responses (Fehres et al., 2013). The coating of therapeutic antigen onto the outer surface MNs has been successfully used for vaccination purposes (Larraneta et al., 2016). Antigen delivery is achieved by diffusion of the antigen from the MN surface following their insertion into the skin, depositing the payload to a depth determined by the MN length and application process (Bal et al., 2010). Silicon MNs coated with a vaccine against malaria showed an efficacy comparable to conventional i.d. injection after challenge with live *Plasmodium berghei* sporozoites (Pearson et al., 2015). Kim et al. revealed that a single dose of an influenza vaccine coated onto stainless steel MNs produces superior immune response compared to i.m. vaccination (Kim et al., 2010). More recently, a solid MN patch coated with influenza virus induced higher Th1 responses, such as IgG2a isotype antibodies and IFN- γ , compared to those induced by i.m. injection (Kim et al., 2015). In another study, stainless steel coated MNs were used to vaccinate against human papillomavirus and stimulated strong protective immune responses (Kines et al., 2015).

This study aims to develop and test, for the first time, a minimally-invasive method of vaccination against *Leishmania spp.* by coating a solid MN device with a plasmid DNA cocktail encoding the nucleosomal histones of *Leishmania infantum*. The study compares the immunogenicity of the MN approach with conventional s.c. and i.d. injection and evaluates the protective capacity in BALB/c mice that have been challenged with *L. major* parasites.

2. Material and Methods

2.1. Animals and parasites

Female BALB/c mice (Harlan, Spain) weighing approximately 20 g and aged 8 weeks were kept under conventional conditions, with unrestricted access to food and water. Animals were housed in groups of 5, in controlled environmental conditions according to ethical standards approved by the Animal Ethics Committee of the University of Navarra and in strict accordance with the relevant European legislation.

The appropriate virulence of *Leishmania major* parasites (clone VI, MHOM/IL/80/Friedlin) was maintained by serial passages in BALB/c mice. Promastigotes were cultured in flasks at 26 °C in continuous stirred Schneider's modified medium (Sigma, USA), supplemented with 20% FBS

and 40 mg/ml of gentamicin (Sigma, USA). *L. major* amastigotes were obtained from popliteal lymph nodes or lesions and, after transformation to the promastigote form, parasites were grown to the stationary phase (5-6 days) before they were used for subcutaneous murine inoculation at the base of the tail.

2.2. Preparation of DNA plasmids and recombinant proteins

The recombinant plasmids used in these experiments (Iborra et al., 2004) (pcDNA3-LiH2A, pcDNA3-LiH2B, pcDNA3-LiH3 and pcDNA3-LiH4) were obtained using the endotoxin-free Giga-preparation Kit (Qiagen, Germany) following the manufacturer's instructions. Purified DNA was re-suspended in phosphate buffered saline (PBS, Gibco, USA) and stored at – 20 °C until use. Expression and purification of the His-tagged recombinant proteins (pQEH2A, pQE-H2B, pQE-H3 and pQE-H4) were performed as previously described (Iborra et al., 2004). After binding to a Ni-NTA agarose column (Qiagen, Germany), recombinant proteins were gradually refolded on the affinity column as described (Shi et al., 1997). Recombinant proteins were eluted with 0.3 or 0.5 M imidazole (Sigma) and dialyzed against PBS. Finally, proteins were passed through a polymyxin-agarose column (Sigma) in order to eliminate endotoxins (endotoxin-free, less than 30 ng/mg of recombinant protein) and kept at – 80 °C until use.

2.3. Microneedle fabrication

Solid MNs were fabricated at Cardiff School of Engineering by cutting needle structures from stainless steel sheets using wire electrical discharge machining (wire-EDM). The steel needles were then electro-polished to debur MN edges and sharpen the tips. The electro-polishing was carried out using a method described previously (Gill and Prausnitz, 2007). Briefly, a 300 mL glass beaker containing a solution of glycerol: ortho-phosphoric acid (85%): deionized water (6:3:1) was stirred (150 rpm) and heated to 70 °C. The cathode was connected to a copper plate and the anode to a MN array. Subsequently, a current density of 1.8 mA/mm² was applied to each MN array for 15 minutes. MNs were cleaned by three immersions in deionized water and then 25% nitric acid for 30 seconds each. This was followed by another washing step in hot water and a final wash in deionized water. A MN applicator capable of concurrent insertion of three planar rows of ten MNs (30 MNs in total) was fabricated from biocompatible acrylate

polymer (e-Shell 200, EnvisionTEC) using an additive manufacturing technique (Zhao et al., 2016).

2.4. Imaging microneedles

The surface morphology of MNs was imaged using a Zeiss Stemi 2000C stereomicroscope associated with an Olympus C3040-ADL digital camera, and was further characterized by scanning electron microscopy (SEM, Veeco FEI XL30, Philips). All pictures were post-processed using ImageJ software.

2.5. Coating microneedles with DNA

MNs were visualized under the stereomicroscope throughout the coating process. A 4.5 μ l (Tris-EDTA (TE) buffer, Qiagen) solution of a plasmid DNA 60 μ g cocktail containing 15 μ g of each recombinant plasmid from *L. infantum* (pcDNA3-LiH2A, pcDNA3-LiH2B, pcDNA3-LiH3 and pcDNA3-LiH4; pHIS) was loaded into a 10 μ l ultra-long tip using a 2.5 μ l pipette. The plasmid DNA cocktail was then deposited onto three rows of ten MN arrays, using a brushing method, reported previously (Zhao et al., 2016), resulting in a total coating of 60 μ g across 30 individual MNs (optimization studies, as shown in Fig. 1f, investigated application of a target dose of 10, 20, 30 or 60 μ g of DNA per 30 MNs). The same procedure was also used to coat 60 μ g of non-recombinant plasmid (4.8 μ l, as negative control; pC). To determine if the coating process affected plasmid stability, the MN coated material was recovered in 150 μ L of TE buffer. Aliquots from the TE buffer were then loaded into a 2% agarose gel supplemented with ethidium bromide and was subject to electrophoresis at 100 V for 1 h. Coating efficiency was calculated by recovering the coated pDNA from the MNs, in 200 μ l TE buffer, for quantitative analysis (NanoDrop®, ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies).

2.6. DNA delivery studies

The 30 MNs coated with 60 μ g of either pcDNA3 WT (negative control, pC) or the plasmid DNA cocktail (pHIS) were applied directly to shaved mouse skin for 5 or 10 minutes. The delivery efficiency was calculated by determining the mass of DNA remaining on the MN device following skin insertion (NanoDrop®).

$\%$ delivered = (calculated mass before delivery – mass post-delivery)/ (calculated mass before delivery) x 100

2.7. Visualization of fluorescence-conjugated DNA delivery into skin

The plasmid DNA cocktail, containing *Leishmania* histones, was fluorescently labelled with Cy3 (Label IT[®] Cy[™] 3 Labeling kit, Mirus Bio LLC) following the manufacturers instructions. 60 µg of the fluorescence-conjugated DNA cocktail was then coated on MNs that were subsequently inserted into shaved mouse skin for 10 minutes before the treated skin was post-stained with methylene blue 20% (w/v), sectioned and fixed in 4% (w/v) paraformaldehyde (Sigma) overnight. Samples were then washed twice with PBS for 30 minutes, embedded in OCT[®] medium and stored at – 80 °C. Skin sections of 10 µm were captured onto Superfrost Plus[®] microscope slides using a cryostat (CM3050S Leica Microsystems), dried overnight and observed with an Olympus BX50 light/fluorescence microscope supplied with URFL-T power and attached to an Olympus DP-10 digital camera. Uncoated MNs were also applied for 10 minutes with subsequent skin staining using methylene blue (20%) to identify MN-created microchannels (Pearton et al., 2012). The skin samples were fixed, sectioned (10 µm) and stained with haematoxylin and eosin (H&E) for histological evaluation.

2.8. Immunization strategies and parasite challenge

Immunizations were carried out in groups of 10 mice with 6 different groups evaluated. The day before each immunization mice were anaesthetized using isoflurane and the upper back (site of injection) was shaved with electric clippers (Contura, Wella). Control groups (pcDNA3; pC) and groups receiving the DNA cocktail (pcDNA3-LiH2A, pcDNA3-LiH2B, pcDNA3-LiH3 and pcDNA3-LiH4; pHIS) were inoculated either s.c. or i.d., at an equivalent DNA dose derived from the MN delivery efficiency studies in a total volume of 30 µl sterile PBS, using 29G insulin needles. For the groups immunized with MNs, the coated MN arrays were mounted into the applicator device, manually inserted into the skin of the mice and held in place for 10 minutes. Mice were immunized three times at three-week intervals between each immunization. Finally, four weeks after the last inoculation, spleens and inguinal lymph nodes for 5 mice were collected for evaluation. While spleens were stimulated to detect cytokine expression, draining lymph nodes were immediately placed in RNA later[®] and stored at -20 °C for further studies. The remaining mice (5 mice per group) were challenged with infective 10⁵ *L. major* metacyclic

promastigotes by s.c. injection in the base of the tail. Lesion size was monitored every week using a digital caliper and recorded.

2.9. Determination of antibody titres and isotypes

Blood samples for serology assessment were obtained by orbital plexus puncture eighteen days after the second and third immunization. Serum samples were analyzed for specific antibodies against *Leishmania spp.* histones. Briefly, high-binding ELISA plates (Greiner Bio-one) were coated overnight at 4 °C with 100 µL of rLiH2A, rLiH2B, rLiH3 or rLiH4 (2 µg/mL in PBS), respectively. In order to determine the titre, defined as the inverse of the highest serum dilution factor giving an absorbance > 0.2, serial dilutions of the sera were performed. The isotype-specific analyses were done using peroxidase-labelled anti-mouse IgG1 (1:1000) or IgG2a (1:500) (Nordic Immunology, The Netherlands) as secondary antibodies. The peroxidase substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid 0.01% (w/v) (ABTS, Sigma) in citrate buffer pH=4 and hydrogen peroxide 0.004% (v/v), was added and the absorbance was recorded at 405 nm after a 30 minute incubation period (iEMS Reader MS, Labsystems).

2.10. Cytokine detection in supernatants of immunized mice

Briefly, splenocyte suspensions were prepared in complete RPMI medium (RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% non-essential aminoacids, 0.5% sodium pyruvate and 0.1% 2-mercaptoethanol). 100 µL of the suspensions (final concentration of $5 \cdot 10^6$ cells/mL) were seeded in 96-well plates (Corning) and stimulated for 48 hours in the presence of the recombinant cocktail (rLiH2A+ rLiH2B+rLiH3+rLiH4, 12 µg/mL of each protein). Culture supernatants were harvested at – 80 °C for analysis. The release of several cytokines related with Th1/Th2/Treg bias was measured in the supernatants of splenocytes cultures by commercial ELISA Kits (BD Biosciences, USA) or Multiplex assays (Milliplex®, Millipore).

2.11. Quantitative real-time RT-PCR

IFN- γ , IL-4, IL-10, IL-12p35, TGF- β , iNOS, TNF- α and β -actin expression were measured in the draining lymph nodes of immunized mice by quantitative real time PCR using iQ SYBR Green supermix (Bio-Rad) and specific primers for each gene (see Table 1) in a CFX96 system from Bio-Rad.

2.12. Parasite load quantification

Animals were euthanized fifteen weeks post-infection and livers, spleens and lymph nodes were aseptically excised. The number of viable parasites was calculated by PCR.

2.13 Statistical analysis

Statistical significance was analyzed using Prism 6.0 software. Differences were tested using the One-Way ANOVA with Dunn's post-test for multiple comparisons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Results

3.1. Coated MNs for the delivery of plasmids into murine skin

Stainless steel solid MNs with the same dimensions and characteristics to those used previously (Zhao et al., 2016) were deburred and sharpened by electropolishing (Fig. 1a and 1b). Scanning electron microscopy (SEM) revealed that the polishing process reduced the MNs length from $477.1 \pm 20.3 \mu\text{m}$ to $404.2 \pm 29.2 \mu\text{m}$. MN protrusions were then coated with either a control plasmid or the DNA cocktail (pC and pHIS, respectively). One row of ten MNs were coated with either 10 or 20 μg and this was then repeated in order to load 30 or 60 μg over three rows of ten MNs (Fig. 1c and 1d). The coating process changed the tertiary structure of the DNA (Fig. 1e). Before coating gel electrophoresis indicated that the pHIS mixture was in the supercoiled form, however after coating an additional band was detected, corresponding to linear DNA (upper band) (Fig. 1e). The mean coating efficiency was between 87 and 99% (Fig.1f). All further experiments were performed with the highest coating dose of plasmids i.e. 60 μg (mean mass of $58.2 \pm 4.8 \mu\text{g}$ for pC and $59.3 \pm 3.4 \mu\text{g}$ for pHIS coated onto MNs, which means a 97% and 98% coating efficiency, respectively) coated onto three arrays and assembled into an applicator device to enable the concurrent insertion of 30 MNs in a single application (Zhao et al., 2016). The dose of plasmid delivered into the mouse skin was dependent on the insertion time (Fig. 1g), with a greater and more reproducible delivery of both pC and pHIS after a 10 minute insertion time compared to a 5 minute insertion time. These experiments predict the dose of plasmid delivered into the skin to be approximately 50 μg for pC

and 55 µg for pHIS. Equivalent doses were therefore administered by conventional s.c. and i.d. injection.

3.2. DNA delivery to ex vivo mouse skin

To confirm successful MN insertion, MNs (uncoated) were applied to skin for 10 minutes (Fig. 2a). Methylene blue staining was used to detect puncture sites from individual MNs (Fig. 2b). Histological analysis of skin sections confirmed MN insertion sites in the mouse skin. MNs penetrated to a depth of approximately 150 µm (Fig. 2c). Fig. 2f shows a 10 µm mouse skin cryosection following treatment with MNs coated with 60 µg of the fluorescently labelled-DNA cocktail (pHIS) (Fig. 2d). After a 10 minute insertion the majority of the pDNA was release from the MNs (Fig. 2e) and plasmid deposition was evident in the uppermost layers of the skin.

3.3. A comparison of the immunogenicity of the *L. infantum* histones-DNA vaccine delivered by different routes

The DNA cocktail of four plasmids encoding each one of the *L. infantum* core histones was used to immunize BALB/c mice 3 times at 3 week intervals. Three different administration routes were investigated, s.c., i.d. or MNs, and results were compared to a negative control. Two weeks after the final immunization, IgG2a-specific antibodies against H2A and H4 *L. infantum* histones were detected in the sera of mice (Fig. 3), although antibodies against H2B and H3 were not detected. Moreover, the levels of IgG2a were greater in the group immunized with MNs compared to conventional i.d. injection, although the differences were not significant ($p>0.05$). Mice immunized by s.c. route did not produce any antibodies. Finally, no IgG1 subclass anti-histone antibodies were detected.

The cellular response induced in mice after immunization with the control DNA (pC) or the DNA cocktail (pHIS) was also evaluated. Four weeks after the last immunization supernatants of splenocytes, stimulated for 48 h with a mixture of the recombinant histone proteins, were harvested and analyzed. Fig. 4 indicates that MN vaccination stimulates greater immune responses than s.c. or conventional i.d. administration. A significant increase in IFN- γ /IL-4 ratio (Fig. 4a, $p<0.01$), IFN- γ /IL-13 ratio (Fig. 4b, $p<0.01$), IFN- γ /IL-10 ratio (Fig. 4c, $p<0.001$) and CXCL10 (Fig. 4e, $p<0.05$) and CCL2 chemokines (Fig. 4f, $p<0.05$) was observed after MN vaccination with pHIS compared to s.c. pHIS. The levels of IFN- γ /IL-4 ratio (Fig. 4a, $p<0.05$) and

the chemokine CXCL9 (Fig. 4d, $p < 0.05$) were also significantly higher than those detected after conventional i.d. vaccination with pHIS. Interestingly, mice immunized with MNs coated with pC and pHIS had significantly higher IL-6 and IL-1 β levels than mice immunized with pC via the s.c. route (Fig. 4g, $p < 0.05$ and $p < 0.05$, respectively; Fig. 4h, $p < 0.01$ and $p < 0.05$, respectively). Regarding cytokine expression in lymph nodes, a significant increase in IFN- γ /IL-4 (Fig. 5a, $p < 0.01$), IFN- γ /IL-10 (Fig. 5b, $p < 0.05$), and IFN- γ /TGF- β (Fig. 5c, $p < 0.05$) ratios was observed after pHIS MN vaccination compared to pHIS vaccination using the s.c. route. Furthermore, the expression of iNOS (Fig. 5e) and TNF- α (Fig. 5f) was significantly increased ($p < 0.05$) after MN vaccination, compared to pC administration via the s.c. route. Conversely, IL-12 levels were very low (data not shown) and IL-12/IL-10 ratio was comparable between all administration routes in both spleens (data not shown) and lymph nodes (Fig. 5d). Finally, no significant differences were observed between the different groups in the production of IL-2, IL-17, CCL5 or CCL3 in the spleens (data not shown).

3.4. Vaccination with the pcDNA3 cocktail, using coated MNs, does not protect mice against L. major challenge

This study also evaluated whether the MN immunization strategy could provide protection against *L. major* infection in BALB/c mice. Four weeks after MN vaccination with pHIS, encoding the *L. infantum* histones mixture, mice were challenged with 10^5 *L. major* stationary promastigotes. There was no reduction in the size of the lesion when mice were treated with pC (control plasmid) administered by s.c. route or pHIS administered by MNs (Fig. 6a). In addition, parasite burden in spleens, livers and lymph nodes was similar in treated (MN pHIS) and control (s.c. pC) mice (Fig. 6b).

4. Discussion

Vaccination is the most important weapon for combating infectious diseases. However, as conventional liquid vaccine injections require trained personal, controlled storage and careful disposal, a number of alternative vaccine delivery technologies have received attention. Given their reduced dimensions, MNs significantly reduce patient pain and apprehension and, more importantly, accurately deposit the vaccine into the epidermis and dermis. In this context, MNs

could facilitate improved targeting of DCs and enhanced immunogenicity, potentially at reduced dose, compared against i.m., s.c. or i.d. injection using classic needle and syringe methods (Fehres et al., 2013). Comparative studies using MNs have demonstrated superiority over i.m. or i.d. vaccination in a number of applications (DeMuth et al., 2014; Hsueh et al., 2017; Kim et al., 2009). In this study coated MNs were used to deliver a vaccine payload in a 'dried form'. This offers practical advantages in terms of stability during transport and storage, and while the laboratory scale manual coating process used in this study is time consuming and labor intensive, automated methods of MN coating provide opportunities for scale up.

Leishmania spp. nucleosomal histones elicit interesting immune responses and have been investigated as potential candidate vaccines to prevent leishmaniasis (Carneiro et al., 2012; Carrion et al., 2008b; Iborra et al., 2004). Herein, MN administration of these histones, in the form of a plasmid DNA vaccine, has been explored and may result in a superior immune response compared to conventional i.m., s.c. or i.d. injection administration. The data presented suggests that the MN immunization strategy for pHIS produces an improvement in immune response compared to conventional delivery methods. This improvement was demonstrated in terms of increased Th1/Th2 cytokines ratio (IFN- γ /IL-4 and IFN- γ /IL-13 ratios) and also Th1/Treg ratio (IFN- γ /IL-10 and IFN- γ /TGF- β ratios) in spleens and lymph nodes (Fig. 4a-c and Fig. 5a-c). It is well established that IFN- γ production is fundamental for the development of a Th1 response. In contrast, IL-4 promotes susceptibility to infection, driving Th2 responses. Similarly, IL-13 might block the onset of Th1 development towards a Th2 bias. Additionally, the immunosuppressive IL-10, produced by Treg cells among other cells, is crucial for parasite survival (Kedzierski and Evans, 2014).

TGF- β has also been shown to inhibit Th1-type immune response, leading to *Leishmania spp.* progression, by blocking IFN- γ , TNF- α and NO production (Kedzierski and Evans, 2014). This study showed that MN immunization with the pHIS mixture results in a cytokine profile indicative of a polarized Th1 immune response, which is essential for the prevention of leishmaniasis. However, low IL-12 production, a crucial cytokine for Th1 cell differentiation, was observed in spleens and lymph nodes of immunized mice (data not shown). IL-12p35/IL-10 ratio in the lymph nodes was also not significantly greater in the groups vaccinated with MNs (Fig. 4d). The modulation of the immune response towards a Th1-type was later confirmed by increased levels

of CXCL9, CXCL10 and CCL2 chemokines after *in vitro* stimulation of splenocytes with recombinant *L. infantum* histones (Fig. 4d-f). These chemokines can be down-regulated by *L. major* as an immune response evasion strategy (Guerfali et al., 2008). Furthermore, CXCL9 and CXCL10 are also implicated in Th1 lymphocyte migration. However, CCL2 plays an important role in early immunity against CL and works in synergy with IFN- γ in order to activate macrophages to kill *Leishmania spp.* parasites. CCL3 and CCL5 production in spleens of immunized mice was comparable between the tested groups (data not shown). CCL3 is associated with a Th2 dominant response whereas CCL5 function presents a critical balance between persistence and resistance in CL (Oghumu et al., 2010). These results also support a preference for a Th1 response. Moreover, the higher expression of iNOS and TNF- α in the lymph nodes after immunization with MNs and the plasmid DNA cocktail (Fig. 5e and 5f) also correlates with high Th1 lymphocyte activation as these are markers of M1 macrophage polarization. The generation of these cytokines by macrophages could be induced by IFN- γ produced by Th1 lymphocytes. However, the significantly higher levels of IL-6 and IL-1 β secreted in the spleens after vaccination with MNs and pHIS, compared to the empty plasmid (pC) delivered via the s.c. route, does not support a clear Th1 response. IL-6 can act as both a pro-inflammatory or anti-inflammatory cytokine, being a susceptible element in CL. Several studies have associated IL-1 β with induction of Th1 responses by activating the inflammasome and promoting IL-12, IFN- γ and NO production (Scott and Novais, 2016). No significant differences were observed in IL-2 and IL-17 in the spleens of the test groups (data not shown). Both cytokines have been implicated in protection or resistance against leishmaniasis (Maspi et al., 2016).

Regarding antibody production, the MNs and pHIS immunization strategy produced low, although detectable, levels of IgG2a against H2A and H4 recombinant histones, which indicates a preference to a Th1 response. Other treatments did not stimulate any antibody response. Indeed, vaccination using DNA elicits a negligible humoral response, biasing towards cellular responses (Aguilar-Be et al., 2005). The humoral immune response is of reduced importance in the protection against *Leishmania spp.* parasite. Although the increased Th1/Th2 lymphocyte and Th1/Treg ratio indicates a predominant Th1 response, which was greater in MN vaccinated mice, this strategy was not able to control *L. major* infection in BALB/c mice. Lesion size and

parasite burden in spleens, livers and lymph nodes were comparable in both vaccinated and non-vaccinated mice (Fig. 6a and 6b). Although the immunization schedule was performed with nucleosomal histones obtained from *L. infantum* and, after that, mice were challenged with *L. major* parasites, the sequences for histones in *L. infantum* and *L. major* have high identity values (Iborra et al., 2004)..

The protective efficacy of *Leishmania* histones administered as a DNA vaccine, either homologous (plasmid DNA only) or heterologous immunization (plasmid DNA + recombinant protein and/or adjuvant) has been previously reported against different species of *Leishmania* with contradictory results. This homologous vaccine was effective in murine models of CL caused by *L. major* (Carrion et al., 2008b; Iborra et al., 2004) and *L. braziliensis* (Carneiro et al., 2012) but it was not able to control infection against VL caused by *L. infantum* in mice (Carrion et al., 2008a) or hamsters (Pereira et al., 2015). However, Baharia et al. found optimum prophylactic efficacy using recombinant histone proteins of *L. donovani* instead of *L. infantum* plasmids in those hamsters infected with *L. donovani* (Baharia et al., 2014). These findings highlight the remarkable differences in disease development and host immune response depending on the immunization strategy, animal model and parasite specie used in the experiment.

Additionally, the route of administration may also influence the effectiveness of a vaccine. While in the studies cited above the plasmid DNA cocktail was administered either i.m. (Carneiro et al., 2012; Iborra et al., 2004; Pereira et al., 2015) or s.c. (Carrion et al., 2008a, b), i.d. vaccination has also been used with recombinant histone proteins of *L. donovani*, in combination with a BCG adjuvant, and conferred a high degree of protection (Baharia et al., 2014). Results using different administration routes and homologous vs. heterologous regimens are therefore not comparable. The number and also the life-cycle stage of parasites inoculated for challenge experiments could be another explanation for the inability of the vaccine to offer protection. It is well-known that selective metacyclic promastigotes are highly infective but stationary-phase promastigotes are also highly virulent due to the presence of apoptotic *Leishmania* parasites that provide survival advantage for viable parasites, favoring evasion from the immune system (van Zandbergen et al., 2006). In this study, we infected immunized mice with *L. major* metacyclic promastigotes, whereas in earlier studies the same number of

parasites (but *L. braziliensis*) plus a salivary gland sonicate were inoculated in the stationary growth phase (Carneiro et al., 2012). In addition to using different parasite species and stages, we performed the challenge with 10^5 *L. major* metacyclic promastigotes compared to the lower doses ($5 \cdot 10^4$ or $3 \cdot 10^4$ *L. major* stationary promastigotes) used in previous reports (Carrion et al., 2008b; Iborra et al., 2004). This high parasite dose does not mimic the natural conditions of infection. Similarly, vaccination with the *Leishmania spp.* chimeral Q protein induced strong protection in dogs after challenging with $5 \cdot 10^5$ promastigotes of *L. infantum* (Carcelen et al., 2009) but was unable to control infection when dogs were challenged with $5 \cdot 10^7$ *L. infantum* promastigotes (Poot et al., 2009). The anatomic site of parasite inoculation might be another factor that affects vaccine efficacy, just as it can determine disease severity and immune responses induced by infection (Baldwin et al., 2003). For challenge experiments in the current study, parasites were inoculated in the base of the tail. However, inoculation in the ear (Carneiro et al., 2012) or footpad (Carrion et al., 2008b; Iborra et al., 2004) has also been performed previously. The dose of vaccine administered to mice could also contribute to the lack of efficacy.

In this study, we hypothesized that it was possible to obtain commensurate or greater immune responses using a reduced dose of DNA vaccine when it is administered via MNs since the device would be better than conventional injections at targeting the payload to DCs. Previous studies with this plasmid DNA cocktail obtained protection against *Leishmania spp.* infection by administering a dose of 80, 100 or 200 μg (Carneiro et al., 2012; Carrion et al., 2008b; Iborra et al., 2004). This study developed a MN device with 30 MNs (10 MNs per row) able to coat 60 μg of plasmid DNA (pC or pHIS). Coating optimization revealed that the entire surface of the MNs was coated with plasmid (Fig. 2d). Attempts to coat greater doses resulted in deposition of the plasmid on the base plate rather than the MN protrusions, which precludes subsequent delivery into the skin. The delivery studies revealed that approximately 55 μg (Fig. 1g) of the DNA cocktail was removed from the coated MN structures after application to the skin, which is 1.5, 2 and 4-fold lower than that delivered in previous studies using comparable plasmids (Carneiro et al., 2012; Carrion et al., 2008b; Iborra et al., 2004). In this study histology indicates that MNs penetrated to a depth of 150-200 μm (Figure 2c). However, it is difficult to obtain accurate measurements for the penetration depth of MNs using traditional histology. This is partly due to

changes in the tissue during histology processing but most importantly due to the mechanical properties of the tissue and its tendency to re-seal following physical insult by the MN. Whilst this study demonstrates that delivery of the DNA cocktail using coated MNs was more effective than conventional s.c or i.d. injection in terms of improved immune response, the dose of vaccine delivered into the viable epidermis and dermis by the MNs may have been too low to confer protection. Future studies should investigate the potential of higher dosing strategies, by increasing the number of MN protrusions (40-50 vs. 30 MNs) or using greater MN lengths (to increase the coating capacity and depth of penetration). Hollow MN delivery systems may also offer an alternative method of delivery that has advantages in terms of dosing capacity, flexibility and reproducibility. These MN delivery systems are particularly suited to DNA delivery as they are able to improve intracellular delivery in the skin using a hydrodynamic mechanism (Dul et al., 2017). They are also more amenable to early clinical testing and scale up as they negate the complexities of MN coating and therefore future studies should investigate their potential in leishmaniasis pDNA immunizations.

5. Conclusions

In summary, this study highlights interesting perspectives for the prevention of leishmaniasis and addresses, for the first time, the use of MNs for *Leishmania spp.* vaccination. The reduced pain and ease of self-administration of MNs compared to conventional injections could be particularly attractive in developing countries, diminishing healthcare costs and improving compliance. WHO and the PATH program (Program for Appropriate Technology in Health), have indicated that more rigorous studies are required to compare vaccinations in terms of doses, routes and number of administrations. Overall, results indicate that although a MN administered *L. infantum* histones-DNA vaccine improved immunogenicity, it did not confer protection against *L. major* challenge in the susceptible BALB/c mouse model. Further studies using MN devices that facilitate higher plasmid loads are warranted. Furthermore, research on the immune mechanisms underlying the therapeutic effect of histones and the influence of type (homologous vs. heterologous) and regimen of immunization, administration of immunoadjuvants and the animal models used are critical aspects to consider in the development of potent vaccines against *Leishmania spp.*

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Figure Captions

Fig. 1. Light microscopy images of MNs (a) before and (b) after electropolishing. The coating technique, (c) and (d), used a fine pipette tip filled with 60 μg of pHIS. Gel electrophoresis of pHIS before and after coating (e). Percentage of pC and pHIS coated onto MNs (f). MNs were coated with pC and pHIS and applied to mouse skin for 5 or 10 minutes. The remaining peptide was dissolved off the MNs and quantified to calculate the percentage delivered into skin (g).

Fig. 2. MN array penetration and formation of microchannels after a 10 minute application into mouse skin (a). Methylene blue post-staining further reveals MN puncture sites following microneedle application (bar=500 μm) (b). H&E stained histological section (10 μm) of mouse skin treated with microneedles and showing the point of microneedle penetration (bar=300 μm) (c). Fluorescent images of MNs coated with the fluorescently labeled pHIS before (d) and after (e) a 10 minute insertion into mouse skin (bar=500 μm). Skin cryosection (10 μm) of *ex vivo* mouse skin observed by fluorescence microscopy after a 10 minute application of MNs coated with fluorescently labelled pHIS (bar=50 μm).

Fig. 3. Specific humoral response induced in immunized mice with the DNA cocktail or the control plasmid administered by s.c. route (s.c. pHIS and s.c. pC), by conventional i.d. injection (i.d. pHIS and i.d. pC) or using MNs (MN pHIS and MN pC). Two weeks after the last immunization, mice were bled and sera were tested by Elisa for anti-H2A, -H2B, -H3 and -H4 antibody responses of both IgG1 and IgG2a isotypes.

Fig. 4. Cytokine production by splenocytes of immunized mice either with the DNA cocktail or the control plasmid administered by s.c. route (s.c. pHIS and s.c. pC), by conventional i.d. injection (i.d. pHIS and i.d. pC) or using MNs (MN pHIS and MN pC). Mice were immunized three times at three-week intervals. Four weeks after the last immunization, mice were sacrificed. After *ex vivo* 48 h splenocytes stimulation with the histones cocktail, supernatants were collected and cytokine levels were determined by Multiplex kit. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Cytokine expression in lymph nodes of immunized mice either with the DNA cocktail or the control plasmid administered by s.c. route (s.c. pHIS and s.c. pC), by conventional i.d.

injection (i.d. pHIS and i.d. pC) or using MNs (MN pHIS and MN pC). Mice were immunized three times at three-week intervals. Four weeks after the last immunization, mice were sacrificed and cytokine expression was determined by PCR. Gene expression has been normalized using β -actine. * p <0.05, ** p <0.01.

Figure 6: Course of *L. major* infection in vaccinated mice. BALB/c mice (n=5) were immunized either s.c with pC or by the use of MNs with pHIS (a). 4 weeks later, mice were challenged with infective 10^5 *L. major* metacyclic promastigotes injected by s.c. route in the base of the tail. After 15 weeks, no differences in lesion size were observed between the two groups. The number of parasites in spleen, liver and lymph nodes was equivalent in vaccinated and control mice (b).

Fig. 1

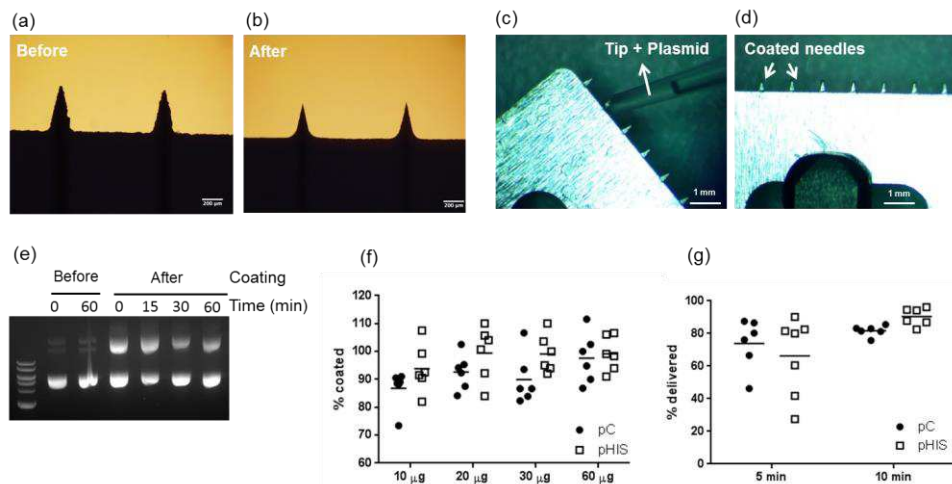


Fig. 2

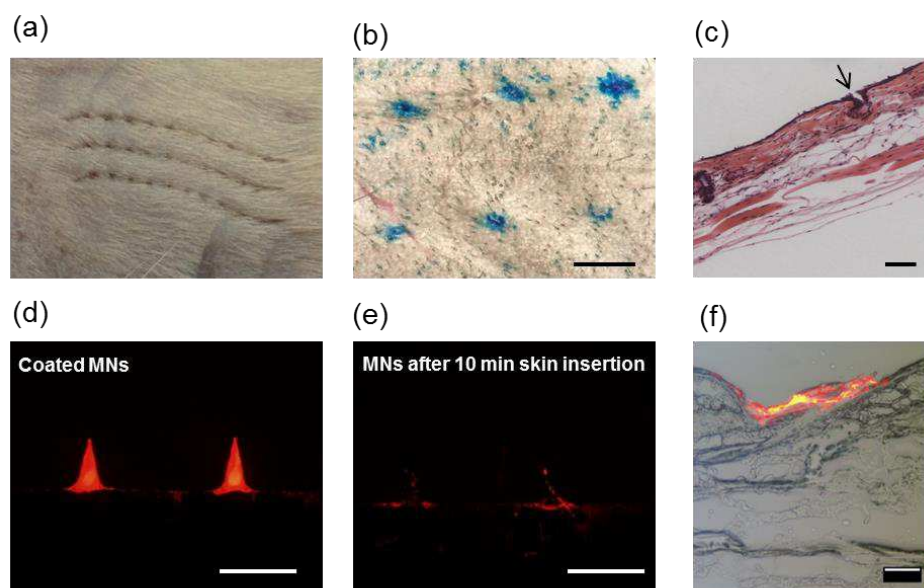


Fig. 3

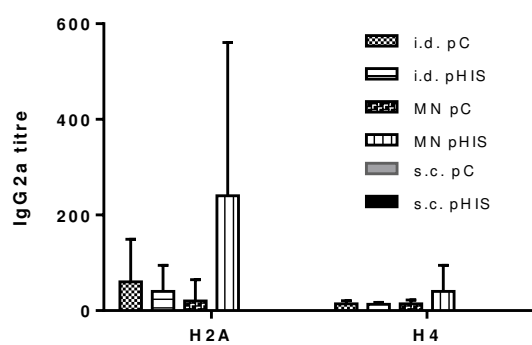


Fig. 4

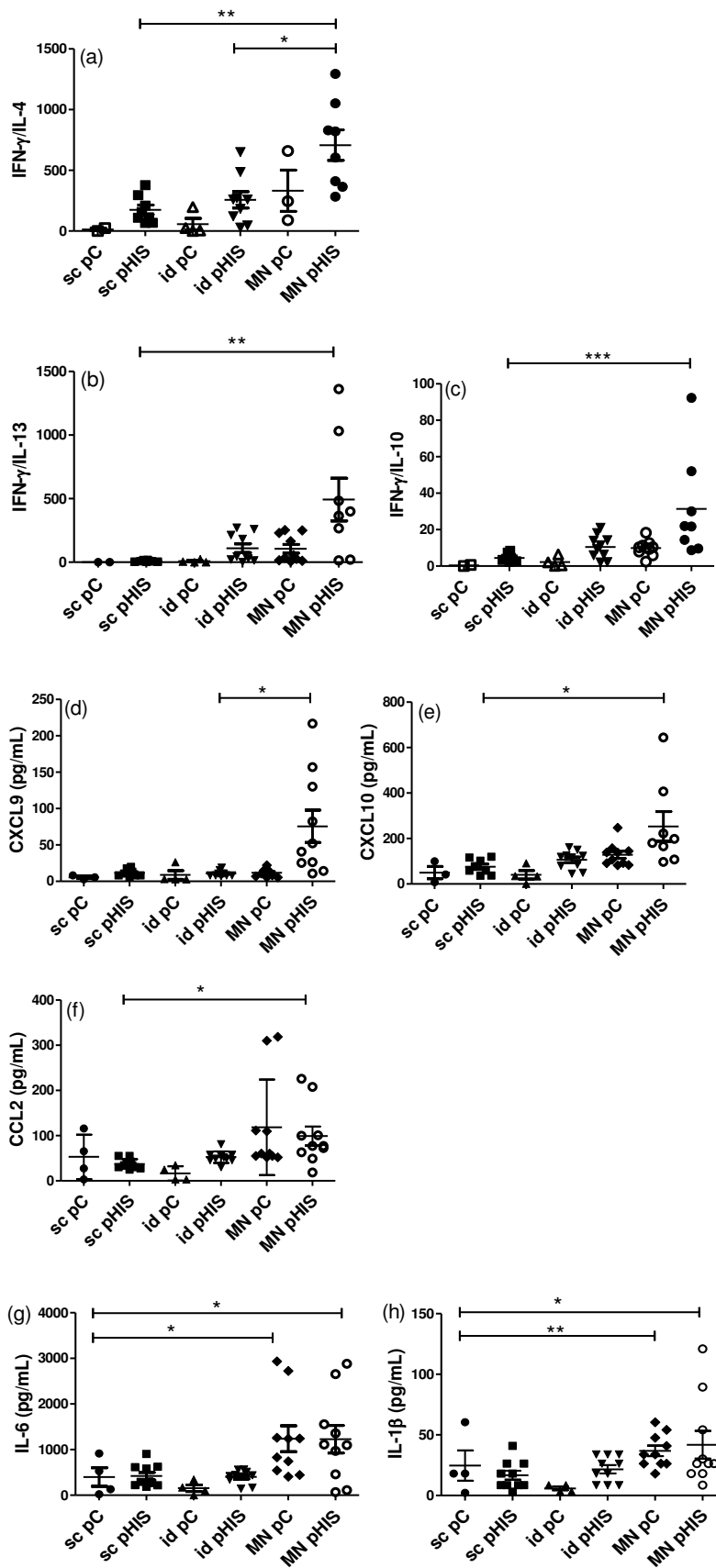


Fig. 5

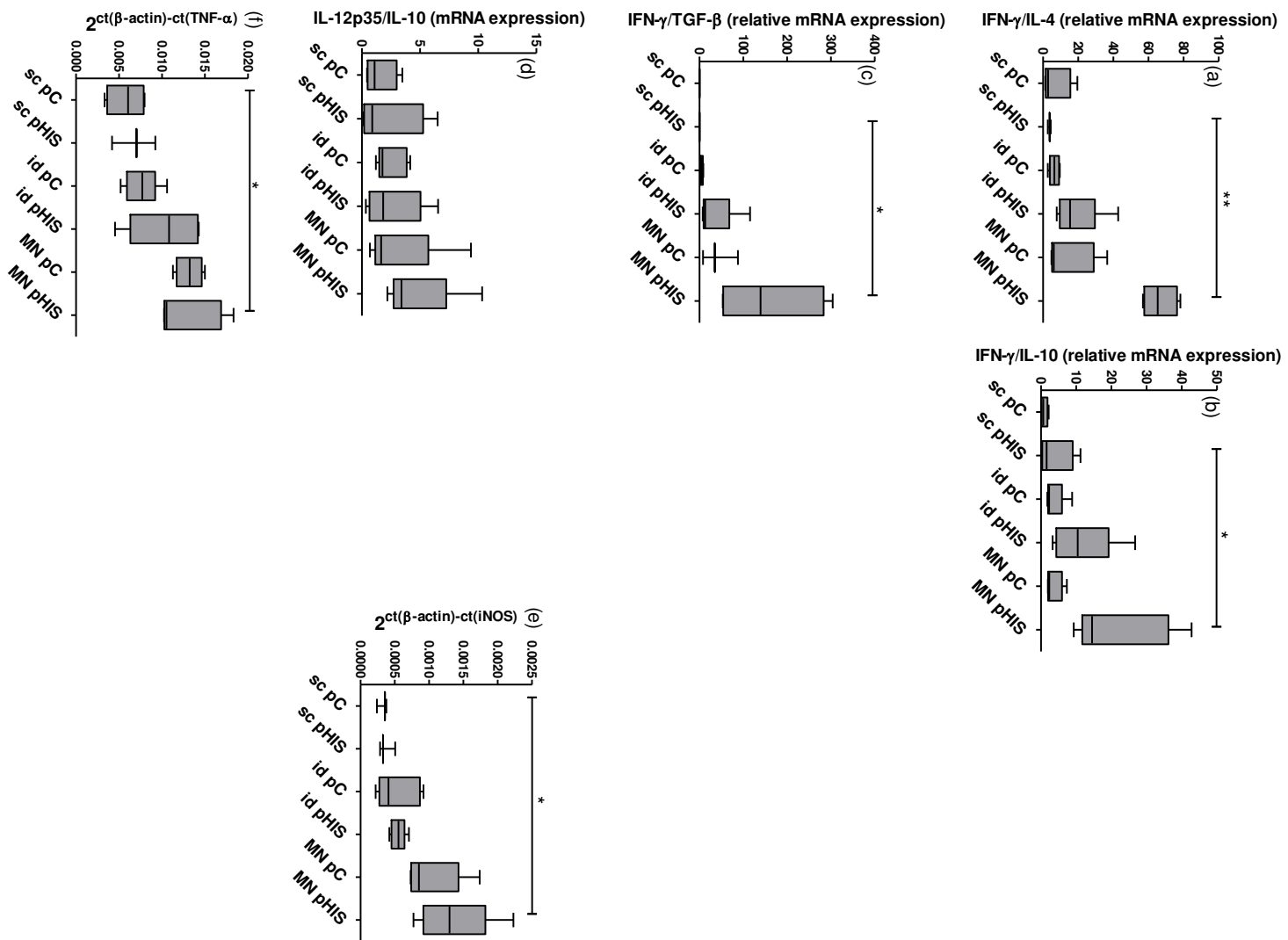


Fig. 6

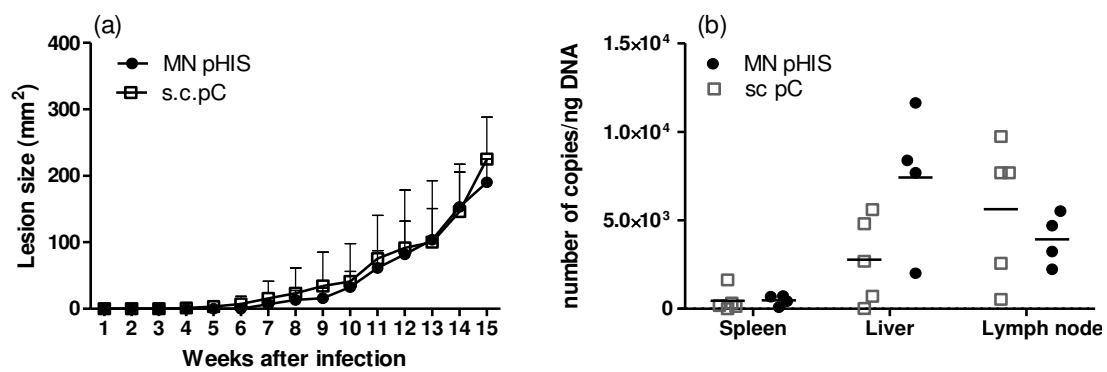


Table 1. Primers used for PCR analysis.

Genes	Nucleotide sequence (5'-3')
IFN- γ	TCAAGTGGCATAGATGTGGAAGAA TGGCTCTGCAGGATTTTCATG
IL-4	GCTATTGATGGGTCTCAACC TCTGTGGTGTTCTTGTTGC
IL-10	GGACAACATACTGCTAACCG AATCACTCTTCACCTGCTCC
IL-12p35	CACGCTACCTCCTCTTTTTTG AGGCAACTCTCGTTCTTGTG
TGF- β	CGGCAGCTGTACATTGAC TCAGCTGCACTTGCAGGAGC
TFN- α	CTTCCAGAACTCCAGGCGGT GGTTTGCTACGACGTGGG
iNOS	TCCTACACCACACCAAAGT AATCTCTGCCTATCCGTCTC
β -actin	CGCGTCCACCCGCGAG CCTGGTGCCTAGGGCG