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DNA immunisation with minimalistic expression constructs

S. Moreno [a], L. López-Fuertes [a], A.J. Vila-Coro [a], F. Sack [b], C.A. Smith [b], S.A. Konig [a], B. Wittig [b,c], M. Schroff [b], C. Juhls [b], C. Junghans [b], M. Timón [a,*]

[a] *Mologen Molecular Medicines S.L., C/Antonio de Cabezón 83, Piso 2, 28034 Fuencarral, Madrid, Spain*
[b] *Mologen AG, Berlin, Germany*
[c] *Department of Molecular Biology and Bioinformatics, Institut fuer Molekularbiologie und Biochemie, Fachbereich Humanmedizin, Freie Universitaet Berlin, Berlin, Germany*

Abstract

The low efficacy obtained in large animals makes plasmid-based DNA vaccines commercially unviable. Another concern is the presence of antibiotic resistance markers on virtually all conventional plasmids. Here we describe the use of minimalistic, immunogenically defined gene expression (MIDGE) vectors for DNA vaccination. MIDGE are linear, covalently-closed vectors containing all the essential information for gene expression and none of the non-essential and potentially dangerous plasmid backbone sequences. MIDGE vectors can also be chemically modified on both ends at defined positions allowing targeting of the DNA to specific cell types or cellular compartments. Immunisation of mice with simple and end-modified MIDGE vectors showed that they are efficacious tools to generate and/or manipulate antigen-specific immune responses.

Keywords: MIDGE; DNA vaccines; Expression vectors

1. Introduction

The use of naked DNA for vaccination holds great promise for preventing an array of infectious diseases for which a suitable vaccine is not available [1]. These include the three big killers (HIV/AIDS, tuberculosis and malaria) responsible for more than 5.5 million deaths per year worldwide [2]. In addition, the same technology has been proposed for its use in immunotherapeutic vaccination protocols against cancer and other devastating diseases [3–5]. The realization that by simply injecting a plasmid, containing a suitable expression cassette, into a mouse muscle led to the uptake of the DNA by the cells and the subsequent protein expression [6] was immediately followed by the demonstration that such approach could also generate a protective immune response [7]. The advantages of DNA vaccination over the rest of the approaches currently in use have been enumerated in several excellent reviews [3–5]. These include the relative ease by which recombinant DNA can be produced, which has allowed a very fast move of the technology from experimental animals to both human and veterinary applications (reviewed in [5,8], respectively). These studies have demonstrated that immunisation with plasmid DNA generates both humoral and cellular immune responses in systems other than mice, although the generally low potency observed has somehow frozen part of the initial enthusiasm. A variety of strategies are being developed to increase the efficiency. Some are aimed at facilitating DNA uptake, such as the use of liposomes [9], in vivo electroporation [10,11], or encapsulation into microparticles [12,13] or bacteria [14,15]. Others are based on the co-injection of plasmids coding for immune-enhancing genes [16,17], or the use of adjuvants such as aluminium salts [18]. Finally, the so-called mixed modality vaccines, consisting on the use of DNA coding for the antigen as a prime followed by a recombinant virus containing the same antigen [19,20], have shown enough potency as to enter clinical trials [21–23]. Although all these approaches have achieved some degree of improvement, the fact is that they also eliminate many of the advantages attributed to the use of naked DNA.

Apart from the efficacy, other issues can hamper the desired transition of the DNA immunisation technology to the clinic. In particular, the inoculation of antibiotic resistance genes, present in all conventional plasmids, into either humans or animals of the human food chain may constitute a major safety concern [24,25].

In summary, there is a need for safe and efficient DNA vectors that can substitute the use of conventional plasmids

* Corresponding author. Tel.: +34-91-728-07-13; fax: +34-91-358-21-60.
E-mail address: timon@mologen.com (M. Timón).

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for immunisation without compromising any of the good qualities associated to them, including their easy manipulation, high stability and simplicity. We have developed minimalistic, immunogenically defined gene expression (MIDGE) vectors that contain only the expression cassette needed for antigen expression in eukaryotic cells and none of the non-essential and potentially dangerous plasmid backbone sequences [26]. In addition, the linear, covalently-closed structure of MIDGE vectors facilitates the binding of molecules such as peptides, proteins, sugars, etc. which could allow the targeting of the DNA to specific cell types or cell compartments and hence increase or modulate the immune response. Here we present data showing that MIDGE vectors are suitable and efficacious tools for antigen expression both in vitro and in vivo, and to generate humoral and cellular immune responses in mice.

2. Materials and methods

2.1. Synthesis of MIDGE vectors

The synthesis of MIDGE vectors has been described elsewhere [26]. Briefly, the gene of interest is sub-cloned into a preparative plasmid containing Eco31I cutting sites flanking the cloning site. The whole expression cassette consists of the CMV early promoter followed by an intron, the gene of interest and a poly (A) signal sequence. After expansion in bacteria, the plasmid is purified and the expression cassette released by digestion with Eco31I. The ends of the expression cassette are protected by the addition of hairpin oligodeoxynucleotides (ODN), specific for both ends, in the presence of T4 DNA ligase (Fermentas, Lithuania). The product is recovered after digestion of all unprotected fragments, including the plasmid backbone, by the $3' \rightarrow 5'$ exonuclease activity of the T7 DNA polymerase (Fermentas), followed by purification.

MIDGE-NLS vectors were produced as follows. The NLS peptide (PKKKRKVEDPYC) was coupled to the hairpin ODN in two steps. First, the amino-modified ODN was activated with sulfo-KMUS (Pierce, Rockford, IL) in PBS at room temperature. After 30 min, the reaction was quenched with TRIS buffer and the activated ODN was precipitated with ethanol. The activated oligonucleotide was resuspended and reacted with peptide at room temperature for 1 h. The reaction was monitored by native electrophoresis through 3% low melt agarose and ethidium bromide staining. The resulting NLS-coupled ODN was purified by HPLC and used to generate the MIDGE-NLS constructs.

2.2. Cell lines

Human chronic myeloid leukemia cell line K562 and human prostate adenocarcinoma cell line DU-145 were obtained from ATCC and maintained in RPMI1640 with 10% fetal bovine serum (FBS).

2.3. Transfection and antigen expression in vitro

For electroporation of K562 cells, 2.5×10^6 cells were prepared in 250 μl serum-free RPMI1640, and combined with 25 ng of DNA in 100 μl of PBS in a 0.4 mm gap cuvette and electroporated with 300 V, 750 μF, and no resistance. Immediately after electroporation, the cells were resuspended in 5 ml complete medium and placed in a 6-well plate and incubated for 24 h. Cells were collected, centrifuged, and the pellet lysed with 100 μl cell culture lysis buffer (Promega, Madison, WI), from which 10 μl was used with the luciferase assay system (Promega). Luciferase activity was measured over 10 s. Transfections were done in triplicate and compared by relative luciferase units.

For electroporation of DU-145 cells, 1×10^6 cells were prepared in 250 μl complete RPMI1640 with 10% FBS, and combined with 40 ng of DNA in 100 μl of PBS in a 0.4 mm gap cuvette and electroporated with 300 V, 750 μF, and no resistance. Immediately after electroporation, the cells were resuspended in 5 ml complete medium and placed in a 6-well plate and incubated for 48 h. Cells were collected, centrifuged, and the pellet lysed with 500 μl cell culture lysis buffer (Promega), from which 15 μl was used with the luciferase assay system (Promega). Luciferase activity was measured over 10 s. Transfections were done in duplicate and compared by relative luciferase units.

2.4. DNA inoculation and antigen expression in vivo

Six-week-old female BALB/c mice were inoculated with the corresponding amounts of plasmid, MIDGE and MIDGE-NLS coding for the luciferase gene under the control of the early CMV promoter. For intramuscular (i.m.) administration, 50 μl of the DNA dissolved in 100 mM NaH_2PO_4 buffer were injected in the tibialis anterior muscle of anaesthetised mice. For the intradermal (i.d.) administration, an area on the back of the mice was shaved and 50 μl of the same solution used above was inoculated in 4 blisters of 12.5 μl each. Seventy-two hours after injection, the whole muscle or the area of skin inoculated were removed, homogenised with a potter in 500 μl of lysis buffer, and the supernatant used to determine the luciferase activity using the enhanced luciferase assay kit (Pharmingen). Luciferase activity was measured for 20 s.

2.5. DNA immunisation

Six to eight-weak-old female BALB/c mice were used in the immunisation studies. Mice were immunised i.d. with 50 μl of a solution containing the corresponding amount of DNA dissolved in 150 mM NaH_2PO_4 buffer as described above. The vectors used for immunisation were plasmid, MIDGE and MIDGE-NLS coding for the surface antigen

of the hepatitis B virus (HBsAg), ayw subtype, under the control of the CMV early promoter.

2.6. Detection of antigen-specific antibodies

Blood was taken at the indicated times post-immunisation by retro-orbital bleeding for the determination of antigen-specific antibodies in sera by ELISA. Briefly, ELISA plates were coated O/N at 4 °C with 150 ng per well of recombinant HBsAg protein, ayw subtype (Aldevron, Fargo, ND). After blocking the plate, 50 μl of 1/2 or 1/4 diluted serum samples was added to each well and incubated for 1 h at room temperature. Alkaline phosphatase (AKP)-conjugated goat anti-mouse total Ig (Southern Biotechnology, Birmingham, AL), or AKP-conjugated goat anti-mouse IgG1 or IgG2a (Pharmingen, UK) was added and incubated for 1 h at room temperature. Plates were reacted with p-nitrophenyl phosphate (Sigma, St Louis, MO) and absorbance read at 405 nm on a Sunrise plate reader (Tecan, Austria). As positive control, 50 μl of a 1/2000 dilution of an anti-HBsAg mouse recombinant antibody (Aldevron) was used.

2.7. Detection of antigen-specific IFNγ-producing CD8+ T cells

Four weeks post-immunisation, two mice per group were culled, their spleens removed and the cell suspension obtained with the help of a 40 μm cell strainer (Becton Dickinson, San Diego, CA). Splenocytes from mice of the same group were pooled together and used on an ELISPOT assay after lysing the erythrocytes, as described [27]. Briefly, 96 wells nitrocellulose plates (Milipore, Spain) were coated with 0.8 μg per well of a purified anti-mouse IFNγ antibody (Pharmingen) by incubating O/N at 4 °C. After blocking the plates, serial dilutions of the splenocytes were added in triplicate. For stimulation, cells from each immunisation group received 2 μg/ml Concanavalin A (Sigma) as positive control or Mytomicin C-treated antigen presenting cells (P815 mastocytoma cell line) either pre-incubated or not with 1 μg/ml of the main antigenic, L^d-restricted, peptide from HBsAg [28]. After 18 h incubation at 37 °C on a 5% CO_2 atmosphere, cells were removed and 0.2 μg per well of biotinilated anti-mouse IFNγ antibody (Pharmingen) added. Plates were incubated O/N at 4 °C and the spots developed by incubating with avidin-peroxidase (Pharmingen) for 1 h at RT followed by the addition of DAB substrate (Sigma). Spots were counted manually with the help of stereomicroscope (Leica, Germany).

3. Results

3.1. MIDGE vectors express genes in vitro at similar levels than plasmid

The expression of genes cloned into MIDGE vectors and plasmid was tested in vitro. The level of expression was comparable irrespective of the cell line used (Fig. 1). Attaching an NLS peptide did not result in an increased expression of the gene.

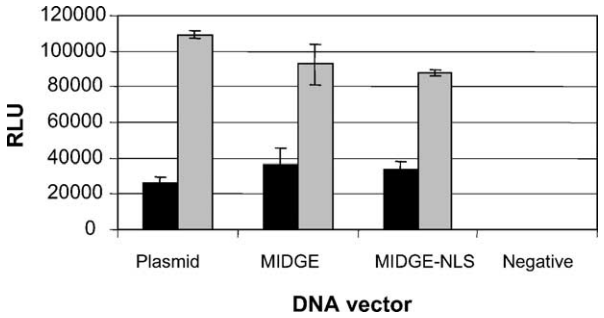

Fig. 1. Expression of antigens coded into MIDGE vectors and plasmid in vitro. The luciferase gene was cloned under the control of the early CMV promoter into a preparative plasmid (pMOK). The resulting plasmid and the MIDGE and MIDGE-NLS vectors derived from it were used to transfect K562 and DU-145 cells by electroporation. Results are presented as the mean±S.D. of triplicate wells. RLU: relative luciferase units. Black columns represent the results of transfecting K562. Grey columns show the result of transfecting DU-145 cells. Negative: untransfected cells.

3.2. MIDGE vectors express genes in vivo at similar levels than plasmid

The expression of genes cloned into MIDGE vectors was also tested and quantified in vivo. Fig. 2A shows a dose–response curve obtained after injection of both plasmid and MIDGE coding for luciferase into the tibialis anterior muscle of mice. The results demonstrate that genes on MIDGE vectors are expressed at levels comparable to those obtained with plasmids over a range of doses. As expected, MIDGE, due to its smaller size and the presence only of the expression cassette, over performs plasmid at some doses (Fig. 2A and B), although this is not consistently observed for all the doses used.

3.3. Attaching a nuclear localisation signal (NLS) to the end of a MIDGE vector does not result in an increased antigen expression in vivo

In spite of the results obtained in vitro, the effect of attaching an NLS peptide to the end of the MIDGE on gene expression was also tested in vivo. As shown in Fig. 2B and C, the expression is not increased by the NLS peptide neither in the muscle nor in the skin. Even more, the luciferase activity was generally lower in organs injected with MIDGE-NLS than in those injected with MIDGE (Fig. 2B and C).

3.4. Immunisation with MIDGE vectors induces the production of more antigen-specific antibodies than immunisation with plasmid

Groups of BALB/c female mice were immunised i.d. with 50 μg of plasmid, MIDGE or MIDGE-NLS coding for

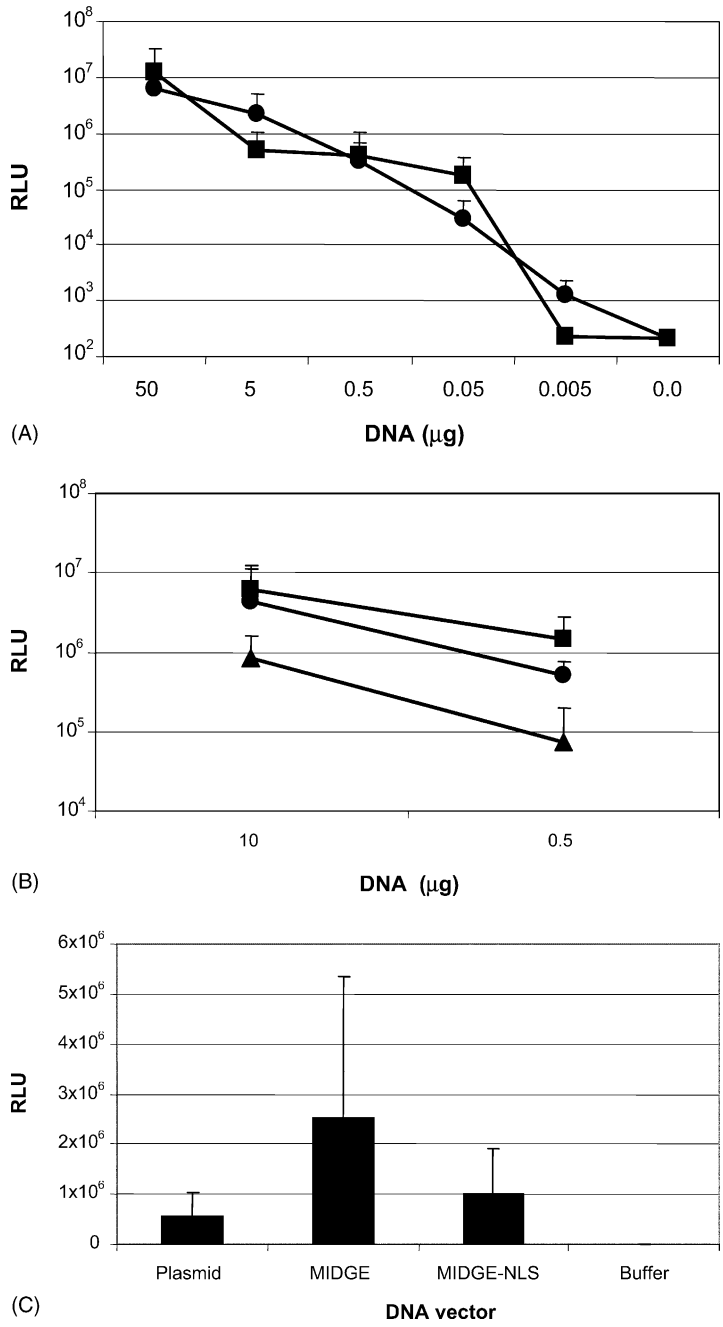

Fig. 2. Antigen expression in vivo. Vectors coding for luciferase were injected into the tibialis anterior muscle or the skin of BALB/c mice and the luciferase activity determined 72 h later in the homogenised tissues. (A) Luciferase activity in muscle after injection of different doses of plasmid (closed circles) or MIDGE (closed squares). (B) Luciferase activity measured in muscles inoculated with two different doses of plasmid (closed circles), MIDGE (closed squares) or MIDGE-NLS (closed triangles). (C) Luciferase activity measured in skin after inoculation with 5 μg of the indicated DNA vector. RLU: relative luciferase units.

HBsAg. Four weeks after immunisation, mice were bled and the presence of anti-HBsAg antibodies was determined by ELISA. Mice immunised with MIDGE-HBsAg had more antibodies than those immunised with plasmid (Fig. 3A). This is probably due to the presence of more antigen-coding DNA/μg. Interestingly, the highest antibody levels were produced by mice immunised with MIDGE-NLS.

3.5. The MIDGE-NLS vector induces antibodies at lower doses than MIDGE or plasmid and the IgG isotype indicates a Th1 shift in the immune response

In order to determine the minimum amount of DNA needed to achieve an immune response in our system, plasmid, MIDGE and MIDGE-NLS vectors coding for HBsAg

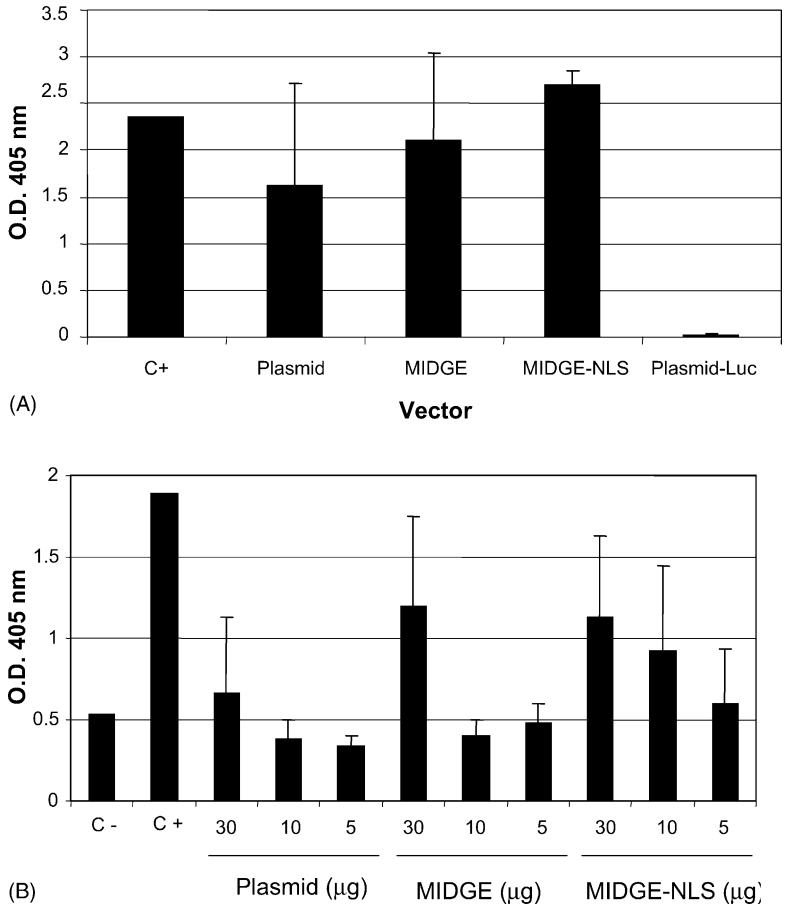

Fig. 3. Antibodies production. The level of antigen-specific antibodies in serum was determined by ELISA 4 weeks after immunisation with different doses of plasmid, MIDGE or MIDGE-NLS vectors coding for HBsAg. (A) Antibody levels after immunisation with 50 μg of the indicated vectors. C+ is an anti-HBsAg monoclonal antibody (clone NF5, Aldevron). Plasmid-Luc: anti-HBsAg antibodies in sera of mice immunised with 50 μg of a luciferase-coding plasmid. Bars represent the mean ± S.D. of 15 mice. (B) Antibody levels after immunisation with the indicated amount of vector. Bars represent the mean ± S.D. of 6 mice. C+ is the same monoclonal antibody used in (A). C−: results obtained in serum from a naïve mouse. O.D.: Optical density.

were administered i.d. at several doses and antibody levels determined 4 weeks after immunisation. Whereas the minimum dose at which plasmid and MIDGE induced antibodies is 30 μg, with MIDGE-NLS we could still detect antibodies at a dose as low as 5 μg (Fig. 3B). The antibodies produced with 30 μg of plasmid are just above the background level, suggesting that this amount is the threshold below which no immune response is generated. However, the level of antibodies induced by the same dose of MIDGE is much higher, presumably again because of the difference in antigen-expressing DNA. The size difference between the plasmid used here (pMOK-HBsAg) and the corresponding MIDGE is 2.42 so the threshold to induce antibodies by the MIDGE vector would be expected to be around 12 μg.

Trying to infer the type of T helper response generated, we determined the ratio of IgG2a/IgG1 antibodies in the sera of mice immunised with the different vectors. Table 1 shows a summary of the results obtained in several experiments. MIDGE-NLS consistently induced a shift towards the Th1 type (high ratio) whereas MIDGE and plasmid did the opposite.

3.6. Immunisation with the MIDGE-NLS vector induces the production of more antigen-specific IFNγ-producing CD8+ T cells than immunisation with plasmid or MIDGE

The generation of antigen-specific CD8+ T cells was determined by an ELISPOT assay 4 weeks after immunisation. The level of INFγ-producing cells was similar in mice immunised with plasmid or MIDGE but consistently higher in mice immunised with MIDGE-NLS (Fig. 4). As shown in the figure, a higher background of cells producing IFNγ was normally seen when the mice had been immunised with

Table 1
Specific IgG2a/IgG1 ratios after intradermal immunisation of mice with DNA vectors coding for HBsAg

Immunising vector	IgG2a/IgG1
Plasmid	0.13–0.22
MIDGE	0.65–0.71
MIDGE-NLS	1.15–1.55

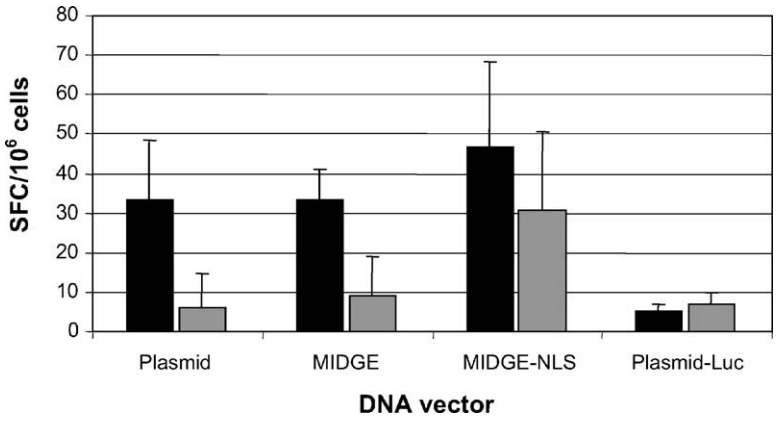

Fig. 4. Generation of IFNγ-producing cells. Four weeks after immunisation with 50 μg of the corresponding vector coding for HBsAg, 2 mice from a group of 15 were randomly selected, the spleens removed and the cell suspension used to determine the amount of IFNγ-producing cells by ELISPOT after re-stimulation in vitro with APC loaded (black columns) or not (grey columns) with an L^d-restricted peptide derived from HBsAg [28]. Plasmid-Luc: cells from mice immunised with a plasmid coding for luciferase. SFC: spot forming cells.

MIDGE-NLS, suggesting the presence of pre-activated cells in the spleen of these mice.

4. Discussion

The urgent need to develop vaccines against diseases for which none of the available approaches is either feasible or effective, has moved the field of DNA vaccination impressively quickly from experimental animals to veterinary and human trials. But the efficacy of the technology has been disappointing in most of the clinically relevant applications, which has prompted researchers to investigate possible ways of improvement. However, in the search for strategies to increase the potency of DNA vaccines, it would be desirable not to lose any of the advantages associated to them. Plasmids normally used for DNA vaccination present several problems that make them inefficient and potentially dangerous for many applications. With this in mind, we have developed minimalistic, immunogenically defined gene expression vectors [26] as an alternative to plasmids for vaccination. Amongst the advantages of MIDGE over plasmids are: (1) its smaller size, (2) the lack of any sequence not required for gene expression in eukaryotic cells, including antibiotic resistance genes, (3) its linear topology, which avoids coiling and so yields a conformationally homogeneous final product, and (4) the possibility of controlling the number of immunostimulatory (CpG) sequence motifs present in the vaccine. In addition, the ends of the MIDGE vectors are accessible to chemical modifications at defined sites allowing the binding of molecules such as peptides, proteins, sugars or other DNA molecules. Here we describe the use of MIDGE vectors for DNA vaccination in mice.

We first demonstrated that MIDGE vectors are as efficient as plasmids for transferring genes into cell lines in vitro. However, the binding of the NLS peptide, which has been shown to mediate the transport of DNA to the nucleus [29,30] and to hugely increase antigen expression [31], did not result in any increase here (Fig. 1). This discrepancy can not be attributed to the variability of the tests, as we have tried many times to reproduce the results published by Zanta et al. [31] always unsuccessfully. Moreover, when gene expression was analysed in two tissues in vivo, again MIDGE-NLS did not show an increased expression (Fig. 2B and C). This is more relevant than the in vitro data published by Zanta et al. [31] because in vivo, particularly in muscle, the number of dividing cells is expected to be very low and so the effect of the active nuclear transport presumably provided by the NLS peptide should be more evident. Genes cloned into a MIDGE vector were expressed in vivo at levels comparable, or even higher, to those cloned into a plasmid (Fig. 2).

To determine the efficiency of MIDGE vectors for immunisation, mice were inoculated intradermally with several doses of DNA coding for HBsAg. At any given dose, the level of antibodies obtained after immunisation with MIDGE is much higher than the one induced with plasmid (Fig. 3A and B). This is likely to reflect the different molarity of the expression cassette, as we have previously demonstrated [32,33]. The level of antibodies obtained with 30 μg of plasmid in our conditions is just above the background level suggesting that the threshold for initiating an immune response must be around this amount. If so, the threshold for the MIDGE vector would be around 12 μg (2.42 times smaller in size) and the 10 μg used in this experiment would be too low (Fig. 3B). More interesting was the observation that the MIDGE-NLS vector induced the highest antibody level at any given dose and that the threshold could be lowered down to 5 μg. Considering that the addition of the NLS peptide never resulted on an increased antigen expression (Figs. 1 and 2), other mechanisms must be operating that explain the increased immunogenicity of this vector. For instance, this structure, consisting on a linear DNA linked to a peptide, could act as a danger signal warning the innate

immune system of the presence of an infection [34]. This is supported by the shift on the T helper response generated by MIDGE-NLS in comparison to MIDGE or plasmid (Table 1). Although it is generally assumed that DNA immunisation always induce a Th1 response due to the presence of CpG motifs, the fact is that many reports have shown that after intradermal immunisation, Th2 is the predominant response obtained, particularly when the antigen is secreted [35,36]. In any case, the final outcome may well depend on the antigen and the plasmid used. In our system, both the plasmid and the MIDGE vector always induced a Th2 response and MIDGE-NLS a Th1. Moreover, these results correlate with our previous demonstration that vaccination with MIDGE-NLS vectors carrying a specific antigen can protect susceptible mice from infection with *Leishmania major* [33], the paradigm of a Th1-mediated protection [37]. Studies are underway to elucidate the exact mechanism explaining the enhanced immunogenicity induced by the MIDGE-NLS vectors.

Finally, the generation of antigen-specific CD8+ T cells was shown to be similar after immunisation with plasmid or MIDGE (Fig. 4) although the dose required for inducing a detectable cellular response was much lower for MIDGE (data not shown). Nonetheless, as observed for antibodies, the best cellular immune response was induced by immunisation with MIDGE-NLS. This protocol consistently gave a high number of IFNγ-producing cells in the absence of peptide, suggesting the presence of pre-activated cells in the spleen of these mice. The nature of these cells (CD4+, CD8+ or even NK) needs to be determined but its presence also underlines the different behaviour of this vector with respect to plain MIDGE or plasmid.

In summary, the data presented here demonstrate that MIDGE vectors are an efficient and safe alternative to plasmids for DNA vaccination. The linear structure of the MIDGE vectors also allows the covalent attachment of different types of molecules, which can be used to target the DNA to specific cells or cellular compartments and, in doing so, increase and/or modulate the immune response. Here we have shown that the binding of an NLS peptide to a MIDGE vector leads to the generation of a stronger and qualitatively different immune response without an increase in antigen expression. This vector was also able to protect mice from *Leishmania major* infection as efficiently as a protocol based on plasmid DNA prime/poxvirus boost [33]. These results suggest that the amount of antigen may not be the only limiting factor to trigger a proper immune response and that the potency of DNA vaccines could also be increased by other means, such as cell targeting or improved delivery.

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