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MINI REVIEW

New insights for the development of efficient DNA vaccines

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Abstract

Despite the great potential of DNA vaccines for a broad range of applications, ranging from prevention of infections, over treatment of autoimmune and allergic diseases to cancer immunotherapies, the implementation of such therapies for clinical treatment is far behind the expectations up to now. The main reason is the poor immunogenicity of DNA vaccines in humans. Consequently, the improvement of the performance of DNA vaccines in vivo is required. This mini-review provides an overview of the current state of DNA vaccines and the various strategies to enhance the immunogenic potential of DNA vaccines, including (i) the optimization of the DNA construct itself regarding size, nuclear transfer and transcriptional regulation; (ii) the use of appropriate adjuvants; and (iii) improved delivery, for example, by careful choice of the administration route, physical methods such as electroporation and nanomaterials that may allow cell type-specific targeting. Moreover, combining nanoformulated DNA vaccines with other immunotherapies and prime-boost strategies may help to enhance success of treatment.

INTRODUCTION

At the latest since the COVID-19 pandemic, nucleic acid-based vaccines that contain antigen-encoding DNA or messenger RNA (mRNA) represent an alternative to conventional vaccines comprising attenuated/inactivated viruses, subunit vaccines, recombinant proteins and cell-based vaccines (Chavda et al., 2021; Ho et al., 2021; Maslow et al., 2023; Poria et al., 2024). The generic, rapid, scalable and cost-effective manufacturing makes them particularly attractive and relevant in situations, where fast and broad availability is required. Compared to protein-based vaccines, transcription/translation-amplified expression of antigen in combination with intrinsic immunostimulatory effects of nucleic acid-based vaccines may be beneficial in terms of efficacy (Liu, 2019). Furthermore, mRNA- and DNA-based vaccines do not only serve as prophylactic and protective immunizations, respectively, against infectious diseases (Lee et al., 2018) but also in the field of cancer immunotherapies (Hager et al., 2020; Lopes et al., 2019), and are employed for immune intervention in autoimmune (Xu et al., 2018) and allergic (Scheiblhofer et al., 2018) diseases. Here, expression of the encoded antigen is intended to induce adaptive immune

responses as required in case of infection or cancer or to inhibit ongoing unwanted auto-inflammatory and allergic reactions, respectively. Table 1 compares the differences between prophylactic pathogen- and therapeutic tumour-directed vaccination.

The antigen-encoding nucleic acid can be either mRNA or DNA, both with specific advantages and disadvantages (Lu et al., 2024; Makker et al., 2024; Pagliari et al., 2023; Poria et al., 2024). The platform-specific advantages and disadvantages of mRNA- and DNA-based vaccines are outlined in more detail elsewhere (Hanke, 2022; Liu, 2019). In comparison to mRNA vaccines, DNA vaccines benefit from (i) higher stability, (ii) greater amplification rates and the option to encode for several antigens, for example, via co-expressed minigenes, at the same time (Tiptiri-Kourpeti et al., 2016) and (iii) the possibility to choose cell type-specific enhancers/promoters to confer transcriptional targeting. However, it has to be mentioned that, in recent years, enormous efforts have been made to improve the potency and stability of mRNA vaccines, for example, by developing self-amplifying mRNA, chemically synthesized minimal mRNA and circular mRNA (Geall et al., 2023; Imani et al., 2024; Perenkov et al., 2023; Zhou et al., 2023).

Regarding the clinical translation of DNA vaccines, regulatory and safety concerns have to be considered (Disis et al., 2023; Myhr, 2017; Stenler et al., 2014). In this regard, despite a potential risk of genomic integration, it was found that, after transfection of plasmid DNA (pDNA), insertional mutations occur less frequently than spontaneous mutations (Ledwith et al., 2000b). On the one hand, long-term persistence and long-lasting expression of DNA could be an issue since for example exogenous DNA was detected up to 6 months in muscle at non-integrated state (Ledwith et al., 2000a). On the other hand, this might also be desired in some indications such as cancer immunotherapy (Liu, 2019). Bacterial sequences within the DNA vector can further induce unwanted immune reactions, but these effects may be strongly reduced by their removal as for instance in minicircle DNA vectors (Stenler et al., 2014). Despite all these possible safety issues, DNA vaccines have proved to be safe and biocompatible in many preclinical and clinical studies, whereas adverse effects were mainly limited to mild reactions at the injection site (Kozak & Hu, 2024).

The history of DNA vaccines goes back more than 30 years. In the early 1990s, Wolff et al. demonstrated in preclinical studies direct gene transfer upon intramuscular application of naked pDNA in mice (Wolff et al., 1990), rodents (Wolff et al., 1991) and non-human primates (Jiao et al., 1992). Nabel et al. (1993) conducted the first clinical study in melanoma patients intratumorally injected with liposomes containing HLA-B7-encoding DNA. Another few years later, DNA vaccines for the treatment of human immunodeficiency virus type 1 (HIV-1) patients (MacGregor et al., 1998) and for malaria prophylaxis in healthy adult volunteers (Le et al., 2000) were tested in phase I clinical trials. Since then, many DNA vaccine approaches have been evaluated in preclinical and clinical trials employing (modified) naked pDNA or more advanced nanoformulations and different administration routes as reviewed elsewhere (Gary

TABLE 1 Distinctions of tumour-targeting and infection-targeting DNA vaccines (Paston et al., 2021; Pollard & Bijker, 2021).

DNA vaccine	Tumour-targeting	Infection-targeting
Antigen	Tumour-associated/specific	Pathogen-specific
Immune response	Primarily cellular immunity (T cell-mediated)	Activation of both humoral (antibody mediated) and cellular (T cell-mediated) immunity
Preventive vs. therapeutic use	Mainly therapeutic	Mainly preventive
Durability of response	May require ongoing treatment for sustained cancer suppression	Long-term immunity with booster doses
Challenges	Identification of tumour-associated/specific antigens; avoiding severe autoimmunity	Identification of highly specific, immunogenic pathogen antigens
Examples	Delayed disease progression in non-metastatic prostate cancer upon treatment with DNA vaccine (pTVG-HP) and nivolumab (McNeel et al., 2023); idiotypic DNA vaccine using PEI for B-cell lymphoma patients (Meleshko et al., 2017); enhanced anti-Lewis lung carcinoma effect of MUC1-VEGFR2 encoding DNA vaccine co-applied with GM-CSF as an adjuvant (Ruan et al., 2017)	Protection of mice against acute toxoplasmosis with a multi-epitope encoding DNA vaccine (Cao et al., 2015); protection against SARS-CoV-2 variants in preclinical models by nanoformulated DNA vaccine (Guimaraes et al., 2024); induction of neutralizing antibodies by a DNA vaccine encoding envelope glycoproteins of two hantaviruses (Hooper et al., 2020)

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; MUC-1, mucin-1; PEI, polyethylenimine; pTVG-HP, plasmid DNA vaccine encoding prostatic acid phosphatase; SARS-CoV-2, severe acute respiratory syndrome coronavirus type 2; VEGFR2, vascular endothelial growth factor receptor 2.

& Weiner, 2020; Kozak & Hu, 2024; Lu et al., 2024; Pagliari et al., 2023). However, so far, the overall success of pDNA vaccines has been rather limited, mainly due to their low immunogenicity in vivo (Pagliari et al., 2023). It has been shown that >95% of pDNA when applied in a non-complexed manner remains extracellular and is rapidly degraded by tissue (endo)nucleases (Barry et al., 1999; Dupuis et al., 2000). The nuclear import of internalized DNA as a prerequisite for transcription to yield mRNA represents an additional barrier in the delivery process, resulting in an overall lower expression efficiency as compared to mRNA vaccines (Liu et al., 2022). In addition, immune evasion processes may hamper the efficiency of DNA vaccines. For example, expression of a DNA vaccine in immunosuppressive cells such as regulatory T cells (Treg), tumour-associated macrophages and myeloid-derived suppressor cells (MDSCs) may induce unwanted immune tolerance (Hager et al., 2020; Shimizu et al., 2018).

Up to now, several DNA vaccines directed against a number of viruses like Ebola (Malik et al., 2023), Zika (Wang, Ling, et al., 2022), hantaviruses (Hooper et al., 2020), HIV-1 (Hou et al., 2021) and human papilloma viruses (Tang et al., 2022) have been positively evaluated in clinical trials. But so far only some DNA vaccines have been licensed for veterinary medicine (Kozak & Hu, 2024; Pagliari et al., 2023), and in humans only the Indian ZyCoV-D vaccine against SARS-CoV-2 has been approved (Blakney & Bekker, 2022; Khobragade et al., 2022). The most critical bottleneck is the inefficient transfection rate in vivo (Jorritsma et al., 2016). Improvement of the in vivo performance of DNA vaccines requires enhancement of their immunogenic potential by (i) optimizing the DNA construct itself, (ii) usage of appropriate adjuvants and (iii) improved delivery to antigen presenting cells (APCs) (Eusébio et al., 2021; Hager et al., 2020; Lee et al., 2018; Li & Petrovsky, 2016). Moreover, combining pDNA nanovaccines with other immunotherapies (Hager et al., 2020; Pagliari et al., 2023) and prime-boost strategies (Guo et al., 2020) may help to enhance the clinical outcome.

This mini-review aims to wrap up the current state of DNA vaccine development for prophylactic vaccinations to prevent infectious diseases and as a therapeutic mean for tumour therapy. The major obstacles for their breakthrough are critically reflected and strategies to improve the immunogenicity of DNA vaccines are discussed with a focus on nanoformulations that allow co-delivery of nucleic acid-based vaccines and, for example, immunostimulatory agents and may enable targeted transfection of APCs to yield pronounced adaptive immune responses. Furthermore, the design of the DNA vaccine itself may be optimized regarding, for example, the deletion of prokaryotic and immunomodulatory sequences, the optimized choice and design of the antigen-coding sequence and the introduction of promoters with cell type-focused activity to achieve transcriptional targeting. Finally, considerations and perspectives for future DNA vaccine approaches are presented.

WORKING MECHANISMS OF DNA VACCINES

DNA vaccines can activate both the innate and the adaptive immune system and depending on their administration route and the transfected cell types, may elicit cellular and humoral immune responses (Eusébio et al., 2021; Hager et al., 2020; Li & Petrovsky, 2016; Pagliari et al., 2023). This may be an advantage over protein-based vaccines, which often fail to induce sufficient T-cell responses (Liu, 2019; Pagliari et al., 2023). The administration route determines which cell types are primarily addressed, and consequently, the main antigen processing pathways (Hager et al., 2020). In this regard, in case of local application of nucleic acid-based vaccines (commonly, intradermal, subcutaneous or intramuscular), keratinocytes (Hengge et al., 1995) and myocytes (Marino et al., 2011), respectively, are the main target cells besides APCs, whereas APCs are rather transfected upon systemic intravenous application (Glass et al., 2016) (Figure 1).

Among APCs, mature dendritic cells (DCs) represent the most potent APC population in terms of T-cell stimulatory capacity (Banchereau & Steinman, 1998; Elwakeel et al., 2023). Intradermal application was found to promote superior immunogenicity over intramuscular and subcutaneous injections (Zhang et al., 2015), which both promoted similar immune responses in clinical trials (Correa et al., 2022), most likely due to the high DC density in the skin, comprising epidermal DCs, termed Langerhans cells and dermal DC populations (Jorritsma et al., 2016). Intravenous application might reach more DCs in secondary lymphoid tissues throughout the body, and consequently yield more antigen-specific T effector cells that home to the target tissue (Zhang, Fan, et al. 2022). This might be especially preferable regarding tumour therapies that aim also on metastases.

In case of transfected non-immune cells (e.g. keratinocytes, myocytes), the expressed antigen has to be released via exosomes or apoptotic bodies to reach APCs (Sudowe et al., 2009). After internalization by the latter, the processed antigen is presented via major histocompatibility complex class II (MHC-II), which in turn leads to the activation of $CD4^+$ T cells. Some DC populations exhibit cross-priming activity and are able to present a fraction of antigen of exogenous origin via MHC-I (Macri et al., 2023). In case of direct APC transfection, the expressed antigen will be loaded onto both MHC-I and MHC-II (Coban et al., 2013; Porgador et al., 1998).

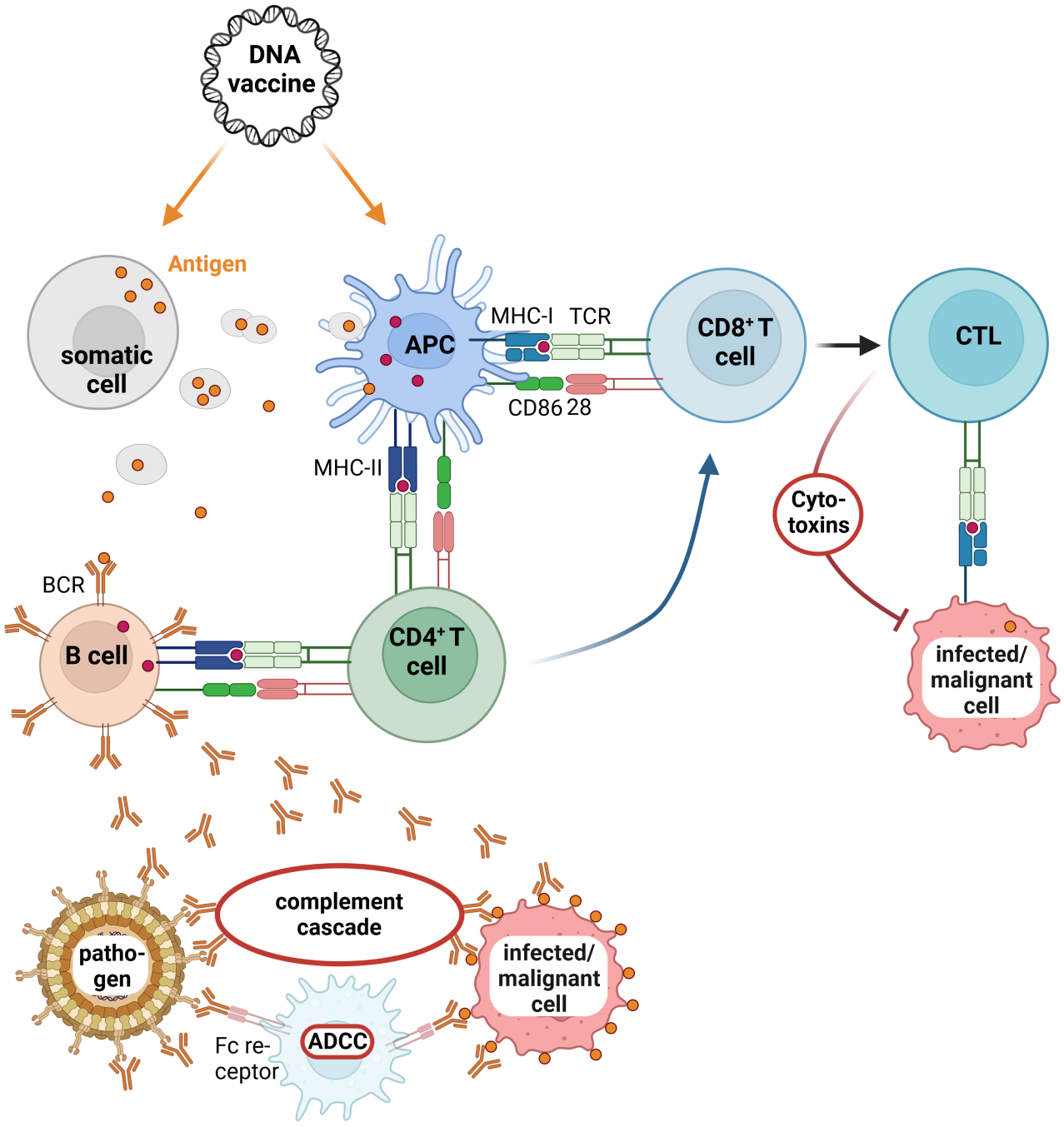

FIGURE 1 Mechanisms of DNA vaccine-induced adaptive immune responses. Upon transfection of somatic cells, antigen (*illustrated as orange dots*) may be released via exosomes or apoptotic bodies that are internalized by antigen presenting cells (APCs). Depending on the delivery route and formulation, a considerable number of APCs may be transfected directly. After processing, protein-derived oligopeptides (*illustrated as red dots*) can be presented both via major histocompatibility complex class I (MHC-I) to $CD8^+$ T cells and via MHC-II to $CD4^+$ T cells, by this triggering T cells whose T-cell receptor (TCR) binds the MHC/antigen complex with sufficient affinity. T-cell activation requires concomitant co-stimulation by APCs via co-stimulatory signals such as CD86/CD28 interaction. Activated $CD4^+$ T cells support the differentiation of activated $CD8^+$ T cells towards cytotoxic T lymphocytes (CTLs). CTLs recognize infected or malignant cells that present the same antigen via MHC-I and kill these by various cytotoxins. B cells are triggered when their B-cell receptor (BCR) engages protein antigen, and pre-activated antigen-specific $CD4^+$ T cells confer B-cell co-activation. Derived plasma cells secrete antibodies, which in turn bind protein on the surface of pathogens and infected or malignant cells. The antibodies' constant Fc part may trigger classical complement activation and via innate immune cells antibody-dependent cellular cytotoxicity (ADCC).

Stimulated APCs upregulate MHC-I and MHC-II surface expression and migrate into secondary lymphoid organs, where T-cell and antibody responses are induced (Hill et al., 2021). Limited antigen presentation via MHC-I and thereby low cellular immune responses are often the reason for low vaccination efficacy

(Comber & Philip, 2014). For a potent CTL response that is particularly relevant in the context of anti-tumour therapies and the killing of pathogen-infected cells, co-activation of $CD8^+$ T cells by T helper subtype 1 (Th1) cells is required (Lu et al., 2021), whereas for a humoral immune response also the Th2 subtype is relevant (Hager et al., 2020; Kozak & Hu, 2024). The polarization into different $CD4^+$ Th subtypes is regulated by the cytokine milieu during T-cell stimulation (Fu et al., 2020). For example, APC-derived interleukin (IL-)12 is important to yield Th1 cells (Farhood et al., 2019). The administration route plays a crucial role as well. Intramuscular injection, for instance, was found to favour stronger Th1-biased immune responses (i.e. CTLs), while intradermal delivery leads to Th2-biased immune reactions (i.e. antibodies) (Hobernik & Bros, 2018; Jorritsma et al., 2016; Kozak & Hu, 2024; Shedlock & Weiner, 2000; Zhang, Zhao, et al., 2022).

Antigen-specific B cells are stimulated to differentiate to antibody-producing plasma cells in case the BCR engages a protein antigen with sufficient affinity (Lam et al., 2020). The B cell is activated by pattern recognition receptors and is co-activated by $CD4^+$ T helper cells recognizing protein-derived peptide antigen presented by the B cell (Kurata et al., 2021). Secreted antibodies may bind surface proteins of pathogens and infected or malignant cells, and targeted killing of antibody-opsonized cells is conferred by complement activation (Sullivan, 2022) and by innate immune cells that recognize the Fc part of the antibodies via Fc receptors and exert several mechanisms like phagocytosis, collectively termed antibody-dependent cytotoxicity (de Vries et al., 2023). B cells can further function as APCs but may require the help of stimulated DCs (Rastogi & McNeel, 2023) to activate antigen-specific $CD8^+$ T cells (Colluru & McNeel, 2016).

Both in case of chronic inflammation and cancer, so-called tertiary lymphoid structures (TLS) may form that play an important role in the induction of T cells and B cells, including plasma cell generation, by this evoking both cellular and humoral immune responses. Further information on TLS is reviewed in detail in Fridman et al. (2023).

Parenteral application of DNA vaccines (i.e. intradermal, subcutaneous, intramuscular and intravenous) mainly elicits systemic humoral immune responses and only limited T-cell-mediated responses and almost no induction of local mucosal immunity (Correa et al., 2022; Zhang et al., 2015). The latter, however, is thought to be particularly relevant for effective prevention of infection and pathogen transmission (Baker et al., 2022; Correa et al., 2022; Knisely et al., 2023; Mostaghimi et al., 2022). Mucosal tissue represents the port of entry and thus the first biochemical (pH, antimicrobial peptides, enzymes), physical (epithelium with tight junctions) and immunological barrier for the majority of pathogens (Song et al., 2024). Mucosal immune defence is thereby mediated by a complex interplay of the innate and adaptive immune system (Brandtzaeg & Pabst, 2004; Correa et al., 2022; Song et al., 2024), comprising tissue-specific immune cells [e.g. alveolar macrophages within the lungs (Hussell & Bell, 2014; Sudduth et al., 2023)], mucosa-associated lymphoid tissue (Brandtzaeg & Pabst, 2004), tissue-resident memory T and B cells for long-term immunity (Knisely et al., 2023; Künzli et al., 2022; Macedo et al., 2024; Mostaghimi et al., 2022; Song et al., 2024) and antigen-specific neutralizing secretory immunoglobulin A (sIgA) as probably the most potent mucosal weapon (Knisely et al., 2023; Song et al., 2024). Also, Th17 cells and IL-17 were reported to be relevant for mucosal immunity by promoting elevated sIgA levels (Baker et al., 2022; Song et al., 2024). To enhance mucosal immunity, alternative application routes to parenteral administration such as direct vaccine delivery to the respiratory [i.e. intranasal or pulmonary delivery (He, Chen, et al., 2023; Knisely et al., 2023; Merkel, 2022; Sudduth et al., 2023)] or gastrointestinal tract [i.e. oral delivery (Correa et al., 2022; Song et al., 2024; Suri et al., 2024)] have come into focus in recent years. In this regard, innovative vaccine formulations such as bioengineered bacteria for oral vaccination (Hu et al., 2020) or spray-dried nano-in-microparticles for inhalation with favourable physicochemical properties for optimal deposition within the lungs (Keil et al., 2019, 2021; Merkel, 2022) as well as application devices like nebulizers or inhalers for intratracheal administration (Sudduth et al., 2023) are required for a potent delivery of the vaccine. Moreover, mucosal adjuvants may increase the immunogenicity of the applied vaccine (Correa et al., 2022; He, Chen, et al., 2023; Song et al., 2024). Combined application routes as accomplished in heterologous prime-boost strategies (e.g. intramuscular plus intradermal or intranasal application) may improve the clinical outcome (Haidari et al., 2017; Künzli et al., 2022; Liu et al., 2023).

To evoke effective induction of antigen-specific cellular and humoral responses, sufficient stimulation of professional APCs is necessary. This can be realized by the use of immunostimulatory adjuvants, which address the innate immune system (Grunwald & Ulbert, 2015; Hager et al., 2020) as outlined below in more detail. A deeper understanding of the interplay between the two branches of immunity is necessary to improve the immunogenicity of DNA vaccines in terms of efficacy and safety.

DELIVERY OF DNA VACCINES

Improved delivery strategies, including viral vectors and nonviral physical and chemical delivery systems, can increase not only the efficiency but also the biocompatibility and safety of DNA vaccines due to dose reduction (Eusébio et al., 2021; Hager et al., 2020;

Hobernik & Bros, 2018; Irvine et al., 2015; Jorritsma et al., 2016; Kozak & Hu, 2024; Lee et al., 2018; Lu et al., 2024). Besides conventional injections, other, less invasive physical delivery methods were successfully tested in preclinical and clinical studies for local application of DNA vaccines, including electroporation (Kisakov et al., 2024), sonoporation (Delalande et al., 2015; Shi et al., 2023), microneedle arrays (Cole et al., 2019; Duong et al., 2018), epidermal tattooing devices (Samuels et al., 2017), needle-free injection systems such as JET injection (Barolet & Benohanian, 2018; Graham et al., 2013; Ledesma-Feliciano et al., 2023) and particle bombardment techniques including particle-mediated epidermal delivery [PMED (Alvarez et al., 2016)] and biolistic transfection via gene gun (Lambracht-Washington et al., 2017; So et al., 2024). Physical stress, for example, in case of electroporation mediated by short electrical pulses, was reported to mediate temporary, reversible cell membrane permeabilization and to induce local inflammatory processes, thereby activating the immune system (Hager et al., 2020; Kisakov et al., 2024). This improved the cellular uptake of the DNA vaccine and yielded stronger immune responses. In case of ZyCoV-D as the only approved DNA vaccine for humans, intradermal delivery is efficiently achieved by a needle-free injection device (Blakney & Bekker, 2022; Khobragade et al., 2022; Sheridan, 2021). For alternative administration routes like pulmonary delivery, innovative devices such as inhalers or nebulizers have been developed, mediating local, efficient application of mucosal vaccines (Sudduth et al., 2023). All of these physical methods can be used to deliver nanoparticles (NPs) as outlined in the following.

Nanoformulations protect nucleic acids from degradation and may target APCs

Viruses have the evolutionary advantage in effective transduction and are characterized by a high transfection rate and intrinsic immunostimulatory effects (Katz et al., 2019; Lu et al., 2024; Travieso et al., 2022). Thus, viral vectors represent highly effective nucleic acid transport vehicles and are used in many preclinical and clinical vaccination studies (Afkhami et al., 2022; Cokarić Brdovčak et al., 2022; Folegatti et al., 2020; Ramasamy et al., 2021; Zhu et al., 2022). However, their disadvantages such as (i) the risk of genome integration; (ii) the sophisticated, difficult production; (iii) a limited cargo capacity; and (iv) off-target immunogenicity and toxicity, call for alternatives. Molecular manipulation techniques (e.g. pseudotyping, self-inactivation or gene elimination) may improve the safety profile (Lu et al., 2024; Travieso et al., 2022). In recent years, nonviral nanoparticulate systems have come into greater focus for nucleic acid-based vaccine approaches including mRNA-delivering lipid nanoparticles (LNPs) that proved to be efficient and safe in humans upon local application (Baden et al., 2020; Polack et al., 2020; Schoenmaker et al., 2021). Such NPs are also suitable for systemic delivery of DNA vaccines by protecting the DNA from degradation by nucleases and facilitating specific cellular uptake into target cells. By now, different NP types have been evaluated regarding their suitability to deliver DNA vaccines, as reviewed in detail elsewhere (Baker et al., 2023; Lu et al., 2024; Mollé et al., 2022; Tang & Li, 2024). Among the various NP carrier systems are polymer- [e.g. polyethylenimine (PEI) (Meleshko et al., 2017; Sun et al., 2021; Zeyn et al., 2023), poly(lactide-co-glycolide) (PLG) (Spearman et al., 2011), poly-D,L-lactic-co-glycolic acid (PLGA) (Li, Xiong et al., 2016), poly(amidoamine) dendrimers (Karpenko et al., 2020; Wood et al., 2005), poly(beta-amino esters) (Andorko et al., 2016; Greenland et al., 2005), chitosan (Wu et al., 2017)], peptide- [e.g. cell-penetrating peptides (CPPs; So et al., 2024)] and lipid-based NPs [liposomes, lipoplexes, LNPs (Guimaraes et al., 2024; Kimura et al., 2021; Liao et al., 2024; Quagliarini et al., 2022; Zhang, Yao, et al., 2022)], as well as inorganic NPs [e.g. iron oxide (Al-Deen et al., 2014; Nawwab Al-Deen et al., 2014), gold (Fogli et al., 2017) and mesoporous silica NPs (Xiong & Qiao, 2016)], extracellular vesicles (EVs)/exosomes derived from various cell types (Cecchin et al., 2023; Dietz et al., 2023; Hagedorn et al., 2024; Kalluri & LeBleu, 2020; Kitai et al., 2017; Lehmann et al., 2023; Rädler et al., 2023), outer membrane vesicles derived from Gram-negative bacteria (Van der Ley & Schijns, 2023) and virus-like particles (VLPs)/virosomes (de Jonge et al., 2007; Gargett et al., 2014). Advantages and disadvantages of the distinct NP delivery systems are summarized in Table 2.

The nanoformulation needs to efficiently compact/encapsulate the DNA and provide extracellular stability but disassemble after cellular uptake, releasing the DNA vaccine in its active form. Bioresponsive elements within the NP such as pH-, redox- or enzyme-sensitive motifs might be helpful in this regard (Hager & Wagner, 2018). The ease and flexibility of modifications, and the possibility to simultaneously load distinct DNA vaccines into one NP and to co-deliver DNA and adjuvants within one nanoformulation, respectively, makes this carrier platform particularly attractive (Ho et al., 2021). Reasonable NP engineering (e.g. formulation conditions, compositional variations or surface modifications) allows to tailor NP properties such as size, surface charge, shape, rigidity and hydrophobicity (Figure 2). Attachment of targeting ligands or shielding agents enables physicochemical passive and ligand-mediated active targeting (Ho et al., 2021; Kim et al., 2023; Steffens & Wagner, 2022).

TABLE 2 Advantages and disadvantages of NPs for DNA vaccine delivery.

Nanoparticles	Advantages	Disadvantages/challenges
Viral vector-based	• High transfection rate • Intrinsic immunostimulatory effects (no need of adjuvants) • Genetic modifications to improve safety profile • Different subtypes with different organ/cell tropism	• Risk of genome integration • Limited cargo capacity • Off-target immunogenicity, toxicity • Pre-existing antibodies • Low efficiency of re-administration • Sophisticated, difficult manufacturing
Polymer-based	• Easy manufacturing by rapid mixing • Rapid self-assembly by electrostatic interactions • High nucleic acid binging capability • Chemical diversity • Tunable particle properties/flexible design • Surface modifications	• 'Polyplex dilemma' (extracellular stability vs. intracellular cargo release) • Challenging scale-up (polydispersity) • Delayed clearance of high molecular weight polymers • Cation-mediated (cyto)toxicity; immunostimulatory potential
Lipid-based	• Easy manufacturing by rapid mixing • Versatile chemistry • Surface modifications • Combinatorial compositions • Encapsulation of both hydrophilic and hydrophobic cargos • Scalability • Good transfection efficiency • Biocompatibility	• Challenging storage conditions • Rather low encapsulation efficiency • Strong hepatic tropism • Immunoadjuvant properties of ionizable lipids
Inorganic (e.g. gold nanoparticles)	• Precise control over size, charge, and surface modifications (reproducible manufacturing) • Possible polymer-coating for improved transfection efficiency • Inertness • Biocompatibility • Optical properties (diagnostics, photothermal applications)	• Non-biodegradability • Prolonged retention, esp. in the hepatobiliary system (toxicity risk)
Extracellular vesicles/ exosomes	• Biocompatibility • Possible modifications • Endogenous targeting; efficient cellular internalization; high delivery rates • Transfection of hard-to-reach targets such as the brain • Low to absent immunogenicity • Prolonged blood circulation times	• Inconsistent yield and loading; rather heterogeneous compositions • Inefficient package of large genes • Demanding large-scale production • High manufacturing costs • Unknown byproducts of donor cells

Note: References: Chen et al. (2022); Irvine et al. (2015); Kim et al. (2023); Lehmann et al. (2023); Lu et al. (2024); Travieso et al. (2022); Uddin et al. (2021) and Wibowo et al. (2021).

The surface characteristics of a NP determine its interaction with blood components and thus the formation of a protein corona in vivo (Berger et al., 2022). This in turn affects the biodistribution, transfection efficiency and biocompatibility. Shielding of the NP surface with, for example, polyethylene glycol (PEG), can reduce protein corona formation (Berger et al., 2022) but may induce unwanted immune reaction in form of anti-PEG antibodies (Zhang et al., 2016). NP formulation under increased salt concentrations (Sasaki et al., 2022), the incorporation of innovative ionizable lipids (Haase et al., 2024), variations of phospholipids (LoPresti et al., 2022) and the employment of a so-called SORT (Selective ORgan Targeting) molecule (Dilliard et al., 2021; Luozhong et al., 2022), respectively, yielded LNPs with selective organ-targeting capability. For example, negatively charged NPs were found to preferably address secondary lymphoid organs (Dilliard et al., 2021; LoPresti et al., 2022; Luozhong et al., 2022). Larger NPs with diameters of around 200–500 nm turned out to be beneficial for targeting of splenic APCs upon intravenous administration (Kranz et al., 2016; Sasaki et al., 2022). Besides that, reticuloendothelial system-mediated clearance was reported to evoke NP accumulation in the spleen (Kim et al., 2023). Passive drainage of NPs into lymph nodes was more effective for NPs with sizes of 10–100 nm (Schudel et al., 2019), whereas bigger particles were largely retained at the application site (Dane et al., 2011).

The size has also an impact on the mechanisms of cellular uptake (Chen et al., 2022; Nguyen et al., 2009; Xiang et al., 2006). Particles with sizes of 0.5–5 μm were mostly internalized by macrophages and immature DCs via macropinocytosis and phagocytosis, while smaller particles entered cells via endocytic pathways (Nguyen et al., 2009; Xiang et al., 2006). In case of endocytosis, efficient endosomal escape is necessary. There exist different mechanisms dependent on the

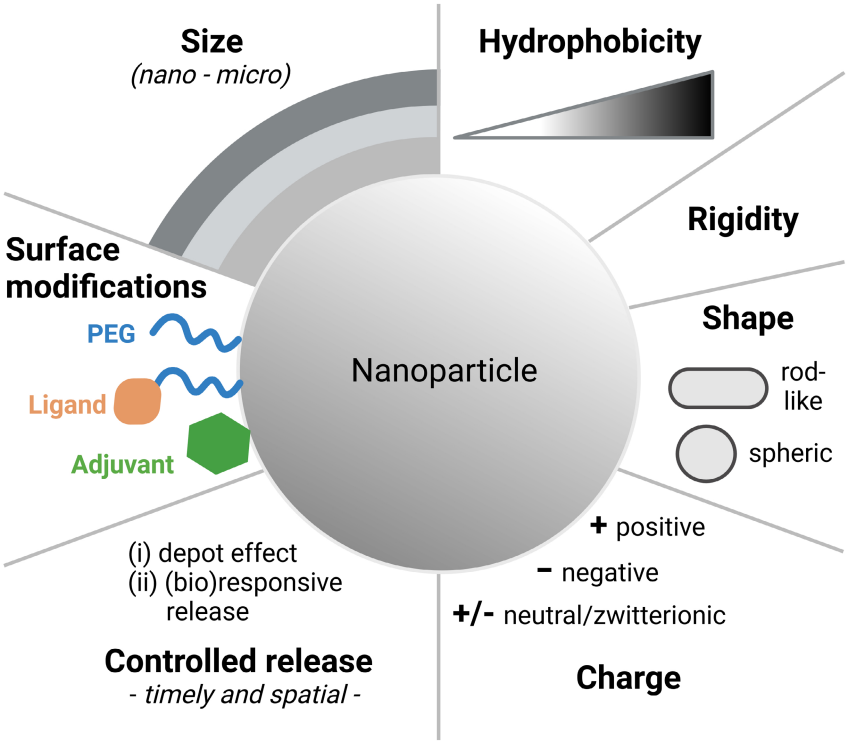

FIGURE 2 Nanoparticle (NP) engineering for improved delivery of DNA vaccines—overview of the various adjustment screws. Passive targeting of NPs is largely determined by their surface characteristics like size, charge and hydrophobicity, all determining the composition of the protein corona in vivo. However, NP surface shielding with polyethylene glycol (PEG) at high density may strongly decrease its formation. Active cell targeting may be achieved by conjugation of a cell surface receptor binding moiety such as an antibody or a carbohydrate. In general, the rigidity and shape of the NP may modulate the extent of its cellular uptake. The bioactivity of the NP's cargo is determined by the time course of release also with regard to endosomal escape. The impact of the different modifications is NP-dependent. Further information as well as examples are provided in the text.

NP type (Bus et al., 2018; Degors et al., 2019; Grau & Wagner, 2024; Hagedorn et al., 2024; Winkeljann et al., 2022). In case of LNPs, for example, membrane fusion and pore formation are considered as the main escape processes. Moreover, the kinetic of the endosomal escape is impacted by the distinct NP types. EVs as intercellular communication vehicles and 'nature's LNPs' (Hagedorn et al., 2024; Lu et al., 2024), for instance, promote up to 10-fold higher escape rates than synthetic LNPs (Bonsergent et al., 2021; Gilleron et al., 2013; Hagedorn et al., 2024; Joshi et al., 2020).

The NP surface charge is further relevant for cellular internalization. Cationic NPs may be internalized more efficiently due to better interaction with the negatively charged cell membrane. However, delivery via negatively charged NPs that bind scavenger receptors present on APCs (Canton et al., 2013; Peiser & Gordon, 2001; Platt & Gordon, 1998; Yu et al., 2015) may be more cell type selective (Kamegawa et al., 2021; Pattipeiluhu et al., 2022). Active APC targeting can be achieved by introducing surface receptor targeting ligands to facilitate enhanced cellular uptake via receptor-mediated endocytosis (Steffens & Wagner, 2022; Wang, Wang, et al., 2022).

So far, the main focus of according approaches have been DCs (Macri et al., 2023). In several cases, the mannose receptor [CD206 (Burgdorf et al., 2006)] and DC-SIGN [dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; CD209 (Appelmelk et al., 2003; Geijtenbeek et al., 2000; Soilleux et al., 2002)], both constituting C-type lectin receptors that recognize mannose, fucose, *N*-acetylglucosamine and mannose-mimicking shikimoyl units, respectively, have been intensively studied (Delehedde et al., 2023; Gao et al., 2020; Moku et al., 2021; Steffens et al., 2024; Sun et al., 2021; Voshavar et al., 2017). Here, it turned out that a high density of mannose units on the NP surface was advantageous (Irache et al., 2008; Mitchell et al., 2001). Especially a trimannose motif was identified to be particularly efficient as shown for example by Wagener et al. (2020) and by White et al. (2006), exhibiting a high affinity towards DC-SIGN (Feinberg et al., 2007). As an alternative to directly target DCs, DNA vaccines that encode an antigen fused with a single chain variable antibody fragment directed against DC-specific surface markers have been investigated in preclinical studies (Chen et al., 2017; Wang et al., 2015). After expression in non-APCs, the secreted fusion protein has been reported to target DCs by binding, for example, DEC205 (Inaba et al., 1995) and CD11c (Villadangos & Schnorrer, 2007), respectively. This

strategy might be a smart way to avoid masking of the ligand with a protein corona, which would otherwise lead to a decreased targeting effect.

Another approach may be to exploit the in vivo formed protein corona to address specific cell types. In this regard, we showed that dextran-coated iron NPs were complement-opsonized upon intravenous application, inducing complement C3-mediated B-cell targeting (Bednarczyk et al., 2021; Shen et al., 2018). Targeting of B cells, which act as APCs and in response to binding of cognate protein antigen to the BCR in combination with sufficient stimulation generate antigen-specific antibodies (Lam et al., 2020), might constitute an interesting vaccination approach for treatment of antibody-dependent diseases like allergy as demonstrated in mouse models of asthma and anaphylaxis (Shen et al., 2018). Here, in a therapeutic setting, co-delivery of a model allergen and of immunostimulatory CpG oligodeoxynucleotides (ODNs) strongly suppressed Th2-dependent allergic reactions due to downregulation of IgE production. The same complement C3 pathway was responsible for preferred expression of tumour antigen-encoding pDNA, encapsulated in LNPs, in splenic B cells, leading to both prophylactic and therapeutic anti-tumoral effects (Kimura et al., 2021).

NPs may exhibit intrinsic immunogenic potential (Chaudhary et al., 2024; Dobrovolskaia & McNeil, 2007; Irvine et al., 2015; Pondman et al., 2023; Sharma et al., 2024; Zhang et al., 2023), for example, by activation of the complement system as outlined above, triggering of the inflammasome or of toll-like receptors (TLRs), or by induction of autophagy. Hydrophobic domains can be recognized as danger signals and initiate innate responses as well (Moyano et al., 2012; Seong & Matzinger, 2004; Shima et al., 2013). On the one hand, this could be exploited as an adjuvant effect to enhance the immunogenicity of DNA vaccines. On the other hand, excessive immune stimulation may raise safety concerns. The cationic polymer PEI, for instance, was found to stimulate the immune system via complement activation (Merkel et al., 2011; Plank et al., 1996) and interaction with TLRs (Chen et al., 2010; Cubillos-Ruiz et al., 2009; Huang et al., 2013). Surface modification or biodegradable cross-linking may reduce these potential immunostimulatory effects of PEI (Hall et al., 2017; Zeyn et al., 2023). For PLG- and PLGA-NPs, activation of the inflammasome has been reported (Demento et al., 2009; Sharp et al., 2009). In case of viral vectors, the immunogenic potential as well as reported pre-existing antibodies (e.g. against the capsid) may be problematic in terms of re-administration (Katz et al., 2019). Furthermore, research has also focused on the development of NPs with directed immunogenic activity (Huang et al., 2022). For example, Anderson and co-workers designed ionizable lipids with a heterocyclic motif for LNP formulations, which promoted potent STING (stimulator of interferon genes)-mediated APC stimulation and increased anti-tumour activity (Miao et al., 2019).

IMPROVEMENT OF DNA VACCINES

Different strategies to enhance the efficacy of DNA vaccines are illustrated in Figure 3 and are outlined as follows.

Prokaryotic parts of the vector backbone limit DNA vaccine activity

A large part of the backbone of conventional DNA vaccines comprises prokaryotic sequences that are required to confer pDNA replication in *Escherichia coli*, and resistance towards a selection antibiotic to prevent loss of pDNA. However, at the same time, the overall length of pDNA has been reported to correlate inversely with the extent of propagation in *E. coli* strains (Yang & Yang, 2012) but also with transfection efficiency in target cells, for example, due to attenuated nuclear translocation (Hornstein et al., 2016). Moreover, cryptic transcription factor binding sites (Newton et al., 2001) and promoter activity (Lemp et al., 2012) within the backbone may interfere with transgene expression. In addition, GC-rich prokaryotic sequences were shown to induce methylation-mediated silencing of transgene expression (Wang et al., 2019). Finally, as described below in detail, sequence stretches of prokaryotic origin may be recognized by danger receptors, thereby evoking immunogenicity (Shirley et al., 2020). Therefore, several strategies have been evaluated to minimize backbone-associated detrimental effects on DNA vaccine efficacy.

Sequence alterations have been shown to prevent transcriptional silencing and to minimize intrinsic immunostimulatory activity (Suzuki et al., 2018). Other strategies focused on minimizing the presence of prokaryotic sequences to counteract size-associated limitations of DNA vaccine usability. To this end, minicircle vectors that harbour recombinase recognition sites at either end of the mammalian transgene expression cassette have been developed (Alves et al., 2021). In *E. coli* strains engineered to express the according recombinase in an inducible manner, first the pDNA is propagated, followed by administration of the recombinase-inducing agent to excise the prokaryotic part of the vector and to religate the eukaryotic transgene expression cassette. The antibiotic resistance encoding part of DNA vaccines has been omitted in so-called nanoplasmid vectors that encode a constitutively expressed silencer RNA-like oligo, termed RNA-OUT (Williams & Paez, 2023). As a prerequisite for nanoplasmid propagation, the bacterial host has been

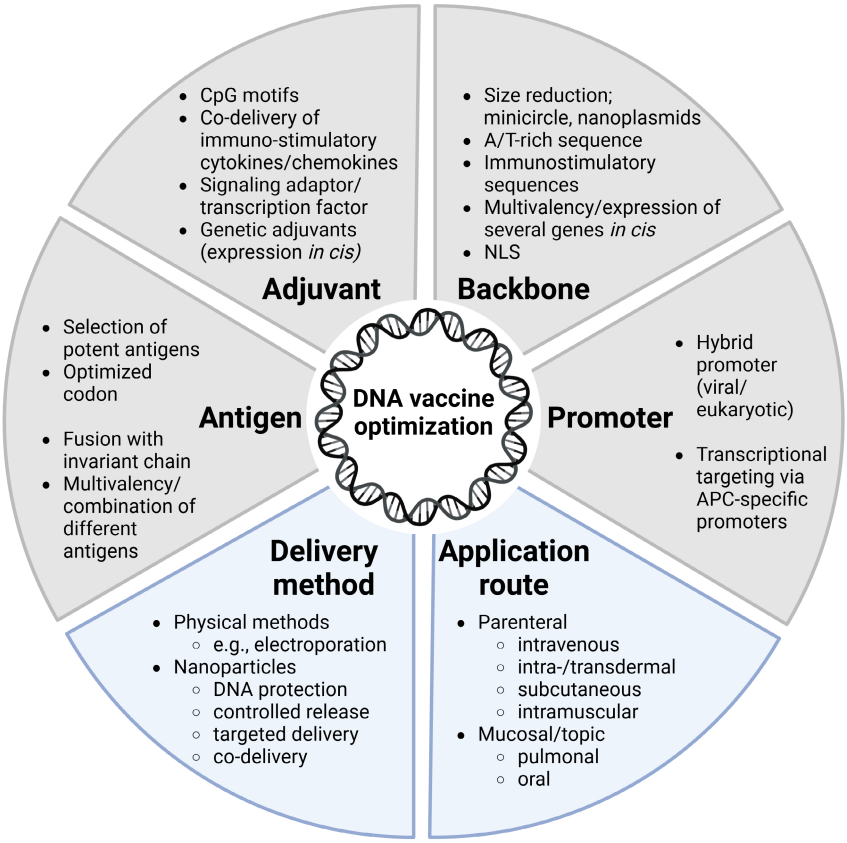

FIGURE 3 Overview of parameters for the improvement of DNA vaccines. Besides optimization of the vector backbone, including the deletion of prokaryotic sequences and the attachment of nuclear localization signals (NLS) to promote nuclear plasmid DNA entry for transcription, choosing a eukaryotic promoter may be important in case that long-lasting transgene expression is required and if transcriptional targeting is intended, respectively. Furthermore, careful selection of proper antigen(s) and the design of the antigen-encoding sequences, for example, in terms of codon optimization are important parameters. The induction and shaping of antigen-specific adaptive immune responses requires co-administration of adequate adjuvants. This can be realized by co-delivery in nanoparticles. The delivery method as well as the application route further impact the efficacy of DNA vaccines. APC, antigen presenting cell.

modified to express an enzyme that forms polymers of exogenously applied sucrose, which are toxic for bacteria. Binding of the RNA-OUT to a complementary sequence stretch of the enzyme-coding RNA prevents enzyme expression. The potency of nanoplasmids to achieve the high levels of immunogenicity in vivo has been demonstrated by Suschak and co-workers (Suschak et al., 2020). Nanoplasmids encoding for a viral antigen and an immunostimulatory RNA expression cassette were applied to mice by intramuscular electroporation and exerted protective effects towards subsequent viral challenge due to strongly enhanced antigen-specific humoral responses.

In general, any DNA vaccine propagated in bacteria requires removal of bacterial components like endotoxins to prevent unwanted side effects (Costa et al., 2023). Therefore, as an alternative, DNA vaccines have been generated by PCR-based amplification of transgene expression units. In this regard, an early study has demonstrated that linear amplicons comprising the mammalian expression cassette were sufficient to yield a cellular immune response in vivo (Sykes & Johnston, 1999). Dumbbell-shaped DNA vectors consist of such linear expression cassettes that are ligated on either end via single-stranded hairpin loops using according enzymes to prevent exonuclease-mediated degradation (Loh & Patzel, 2023).

Besides optimization of the DNA vaccine in terms of size and sequence, the transfection efficiency may be enhanced by tagging either the DNA (Loh & Patzel, 2023) or the complexing NP (Bitoque et al., 2021) with a CPP that promotes cellular entry and endosomal escape (Sun et al., 2023). Furthermore, either entity may be coupled with a nuclear localization signal (NLS) that confers active nuclear entry (Nie et al., 2023), respectively. As a further development, several peptides have been generated that exert both CPP and NLS functions to increase the overall transfection (Li, Hao, et al., 2019).

Eukaryotic promoters confer sustained antigen expression

Plasmids used to drive eukaryotic transgene expression are normally equipped with a virus-derived

promoter that mediates ubiquitous high-level expression as, for example, the Simian virus (SV)40 promoter (Grubor-Bauk et al., 2016) and the human immediate early Cytomegalovirus (CMV) promoter (Tretyakova et al., 2020), respectively. However, it has been frequently observed that these promoters are silenced over time by methylation of CpG islands and histone deacetylation, resulting in chromatin condensation as an innate anti-viral response (Grassi et al., 2003). Eukaryotic promoters that are normally less active than virus-derived ones as well as viral/eukaryotic hybrid promotors, developed to maintain high-level activity, were reported to be resistant towards silencing and to confer sustained transgene expression. Examples for such hybrid promoters are the so-called CAG promoter, which is composed of the CMV enhancer region and the chicken beta-actin promoter (Hollidge et al., 2022), as well as a composite promoter composed of the muscle-specific creatine kinase gene enhancer fused to the SV40 promoter (Takeshita et al., 2007).

Conventional DNA vaccine application approaches like intramuscular injection result in antigen production by non-APCs, and antigen is released by these in form of vesicles (Konishi et al., 2003). These are internalized, processed and presented by APCs (Shakushiro et al., 2004). However, one important common immune evasion strategy of pathogens that may cause chronic infection like the Hepatitis B virus (Li, Yang, et al., 2019), *Mycobacterium tuberculosis* (Cardona & Cardona, 2019) and in case of tumours (Glasner et al., 2023) is to induce and expand immunoregulatory cell types (e.g. Treg and MDSCs) that inhibit innate and adaptive immune cells, including APCs (Haist et al., 2021). Interestingly, also ageing-associated immunosenescence affects the immune system in a similar manner (Hieber et al., 2023). Therefore, both in case of therapeutic vaccines that aim to induce an immune response during an ongoing disease (Lopes et al., 2019) but also in case of prophylactic vaccination of elderly (Tanner et al., 2021), it may be advantageous to restrict transgene expression to professional APCs like DCs (Wculek et al., 2020) and B cells (Rastogi et al., 2022). Coupled delivery of an adjuvant (see below) may overcome a pro-tolerogenic state of APCs and result in sustained adaptive antigen-specific immune responses (Kaps et al., 2023).

As outlined above, APC-focused transgene expression may be accomplished by cell type-specific transfection using, for example, nanoformulations that are conjugated with targeting moieties (Alam et al., 2021; Chen et al., 2017) that engage endocytic receptors on APC or by passive APC targeting achieved by physicochemical NP surface characteristics (Kranz et al., 2016). As an additional level of regulation, transcriptional targeting of APCs may be achieved by using cellular promoters that are predominantly active in these cell types. In this regard, several promoters of genes predominantly expressed in DCs as the most potent type of APCs (Elwakeel et al., 2023) have been used as, for example, dectin-2 (Bonkobara et al., 2001; Morita et al., 2001), CD11c, Langerin, DC-SIGN and DC-STAMP (Moulin et al., 2012). Moreover, we have shown that, within the immune compartment, expression of the murine (Ross et al., 1998) and human (Ross et al., 2000) fascin-1 (Fscn-1) gene is largely confined to DCs. Aside from that, Fscn-1 was found to be expressed by neuronal cells (Rajan et al., 2023) as well as endothelial cell populations (Bai et al., 2023) and metastasizing tumours (Sarantelli et al., 2023). We demonstrated that high-level expression of the human Fscn-1 gene depended on an enhancer region located upstream of the core promoter (Bros et al., 2003). More recently, we reported that fusion of this enhancer region to the core promoter even increased its activity specifically in a DC-like cell line as compared to other cell types in vitro (Zeyn et al., 2023). In addition, this optimized Fscn-1 promoter induced stronger splenic reporter expression than the CMV promoter when administered intravenously after condensation with succinylated branched polyethylenimine (Zintchenko et al., 2008) that was reflected by higher transgene expression in splenic DCs (Zeyn et al., 2023). In other studies, we employed the murine Fscn-1 promotor to drive expression of antigen in DNA vaccines applied by PMED and observed that the induced immune responses were of similar intensity as compared to DNA vaccines containing the CMV promoter (Höhn et al., 2013). Furthermore, whereas CMV promoter-driven immune responses preferably induced Th2 responses, the Fscn-1 promoter evoked Th1-biased adaptive immune reactions (Sudowe et al., 2006, 2020), and immunization with either promoter yielded largely comparable CTL induction (Ross et al., 2003; Sudowe et al., 2003).

Tumour- and pathogen-targeting vaccines differ in their antigen requirements

Nucleic acid-based vaccines contain an expression cassette that encodes a pathogen- or tumour-derived protein or peptide, which may serve as a so-called antigen to induce antigen-specific adaptive immune responses (Beck et al., 2021; Liu, 2019). Expression of the whole protein is necessary to induce protein antigen-specific antibodies (Ulrich-Lewis et al., 2022), whereas derived antigenic peptides are sufficient to trigger $CD4^+$ and $CD8^+$ T-cell responses (Liebscher et al., 2021). The choice of antigen varies notably between cancer and pathogen targets, each requiring distinct considerations for optimal immune recognition and response. Distinctions between tumour- and pathogen-targeting DNA vaccines are summarized in Table 1.

Concerning the design of pathogen-directed vaccines, the antigen must be highly specific to prevent immune reactions against self-antigens (Pollard & Bijker, 2021). For this, suitable antigens may be identified using bioinformatic approaches (Poria et al., 2024) and mass spectrometry-based immunopeptidomics (Mayer et al., 2022), allowing rational vaccine design (Poria et al., 2024; Rueckert & Guzmán, 2012). In this context, conserved or polyvalent epitope sequences are preferable to prevent immune evasion of pathogen variants, as occurring, for example, in case of viruses that show high level of mutations like HIV-1, or influenza viruses (Kozak & Hu, 2024; Poria et al., 2024; Sia et al., 2021). In general, prophylactic vaccines focus to induce pathogen-neutralizing antibodies to protect against infection (Burton, 2023). The antibody-opsonized pathogen may be killed due to classical complement activation (Kemper et al., 2023) or immune cell-dependent pathogen killing (Charles et al., 2022) via recognition of the exposed constant Fc part of the pathogen-opsonizing antibodies (i.e. ADCC). In contrast, therapeutic vaccines primarily aim to induce cellular immune responses against infected (Tang et al., 2022) and malignant cells (Saxena et al., 2021), respectively.

With regard to the latter, neoantigens that arise from mutations unique to tumour cells constitute optimal targets, since these exhibit minimal resemblance to self-antigens (Chong et al., 2022), thereby avoiding the need to overcome self-tolerance (Zhao et al., 2021). Associated with this, targeting of tumour-specific neoantigens, in contrast to tumour-associated antigens that are expressed by non-malignant cells as well, may result in autoimmune reactions due to the activation of auto-reactive B cells and T cells (Jiang et al., 2019). The identification of neoantigens requires patient-specific whole exome sequencing of tumour and non-malignant cells (de Sousa et al., 2021). Hu et al. (2021) demonstrated the presence of persistent memory T cells specific to neoantigens up to 4.5 years following neoantigen vaccination, suggesting the potential efficacy of neoantigen-driven immunotherapy in providing protection against and even control of metastases. One example of a tumour-associated antigen is prostatic acid phosphatase (PAP), which is expressed exclusively in prostatic cells. Treatment of castration-resistant prostatic cancer patients with a PAP-encoding DNA vaccine yielded significant clinical responses when co-applied with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant (McNeel et al., 2009), or the immune checkpoint inhibitor nivolumab (McNeel et al., 2023) to enhance tumour immune cell infiltration.

Therapeutic vaccines may also target immunoregulatory cell types that are induced and expanded both upon chronic infection (Dorhoi & Du Plessis, 2017; McManus & Maizels, 2023) and cancer (Haist et al., 2021), limiting innate and adaptive immune responses by various mechanisms. In this regard, Treg (He, Miao, et al., 2023) and MDSCs (Stevenson et al., 2022) constitute suitable target cells. For this, proteins that are specific for these cell types are chosen as a source of antigen. For example, Treg and MDSCs both express indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan and thereby inhibits the expansion of activated T cells (Adu-Gyamfi et al., 2019). By now, several preclinical and clinical studies have confirmed efficacy of IDO-specific anti-tumour vaccines (Lorentzen et al., 2023; Nandre et al., 2022).

Codon optimization may amplify expression of the encoded antigen and consequently boost the immune response (Peng et al., 2021). Furthermore, modification of the peptide sequence may improve its binding to MHC and thereby enhance the interaction between the MHC/peptide complex and the T-cell receptor, resulting in improved T-cell activation (Yin et al., 2011). In this regard, computational approaches including various modelling techniques and in silico simulations may significantly contribute to identify epitopes with optimal binding affinities to TCRs and BCRs (Poria et al., 2024). Increased antigen presentation via MHC-II may be achieved by fusing its coding sequence with that of the invariant chain (Afridi et al., 2016).

All in all, immunogenicity, influenced by the ability of antigens to induce an immune response, is essential for vaccine efficacy. DNA vaccines, while promising due to their stability and ability to mimic natural infections, face challenges in achieving optimal immunogenicity in human trials (Kutzler & Weiner, 2008; Pagliari et al., 2023). Nonetheless, several strategies have been developed to enhance immunogenicity of DNA antigens, as outlined in this mini-review. As discussed above, the usage of efficient promoters and coding optimization constitute means to improve DNA vaccine efficacy. For example, the number of immunogenic CpG motifs in a gene sequence that are recognized by TLR9 in B cells, macrophages and DCs can be varied to increase effective immune response (Lopes et al., 2017). Besides, soluble or genetic adjuvants (details see below) as well as delivery strategies such as electroporation and NPs that have been outlined above, are verified methods to increase immunogenicity (Shah et al., 2015). In addition, various delivery routes like intradermal or mucosal application aim to enhance immunogenicity by improving antigen uptake and presentation, as highlighted above. Furthermore, various strategies such as microneedles and bacterial vectors showed promise in overcoming these challenges, offering potential to enhance both humoral and cellular immune responses (Porter & Raviprakash, 2017).

Adjuvants shape adaptive immune response

Adjuvants are defined as compounds that increase the reaction of the immune system towards an antigen. Most often, this term is used to describe immunostimulatory

agents that are required to induce adaptive immune responses. However, adjuvants may also serve to induce tolerance (Durham & Shamji, 2023). In general, adjuvants may strongly affect effector functions of innate immune cells (Zhao, Cai, et al., 2023).

In case of vaccinations intended to induce T effector cells and antibody production, adjuvants are applied that mimic an infection or inflammation by engaging danger receptors expressed by innate immune cells (Georg & Sander, 2019). In case of DNA vaccines, nucleic acid-derived adjuvants are of considerable interest since these may be integrated into an antigen-encoding DNA vaccine to ensure co-delivery into the same APCs (Colombani et al., 2023). However, co-delivery may also be achieved using nanoformulations such as LNPs that contain both the DNA vaccine and the adjuvant (Francis et al., 2020). Table 3 depicts the characteristics of adjuvants that are used to enhance the immunogenicity of DNA vaccines.

DNA itself contains intrinsic immunogenic elements that may be recognized by the innate immune system as pathogen-associated molecular patterns (PAMPs) like cytosolic double-stranded DNA, which activates the cGAS (cyclic GMP-AMP synthase)/STING pathway and CpG motifs that trigger TLR9 (Baker et al., 2023; Eusébio et al., 2021; Lee et al., 2019; Li & Petrovsky, 2016; Ori et al., 2017). CpG motifs are specific DNA sequences that are rich in unmethylated cytosine and guanine and thereby mimic bacterial DNA motifs (Goonewardene et al., 2020). TLR9 that is expressed in endolysosomes by innate immune cells as well as B cells recognizes these motifs (Fehér, 2019), which in turn triggers innate immune responses and promotes APCs to confer Th1 polarization (Kocabas et al., 2020) and CTL induction (Xu et al., 2023). Interestingly, the backbone of various plasmid vectors was reported to exert stimulatory effects due to the presence of a CpG-rich motif located in the ampicillin resistance gene (Jiang et al., 2006). To further enhance adjuvancy, additional CpG motifs may be incorporated (Coban et al., 2005). However, it has to be considered that CpG-rich motifs were found to differ in their stimulatory potency and signalling induction in a cell type- (Martinson et al., 2007) and species- (Verthelyi, 2006) specific manner. CpG ODNs are used as the adjuvant component of an approved hepatitis B vaccine (Lee & Lim, 2021) and has been tested in a number of clinical trials assessing the efficacy of vaccines to evoke pathogen-specific (Kayraklioglu et al., 2021) and anti-tumour immune responses (Dongye et al., 2022).

Besides CpG motifs, also pathogen-derived DNA sequences that engage cytosolic DNA sensors (Zahid et al., 2020) may exert immunostimulatory activity by activating the STING pathway (Zhang, Zhou et al., 2022). STING signalling induces interferon-regulatory factor3-dependent expression of inflammatory genes including type I interferons (IFN) that are vital for antiviral responses (Mesev et al., 2019). Moreover, STING activating adjuvants have been reported to promote anti-tumour responses in preclinical studies and clinical trials on various levels, by elevating tumour antigen presentation, enhancing T-cell activation and facilitating tumour infiltration by T effector cells (Ulrich-Lewis et al., 2022).

RNA-based sequences that trigger other danger receptors, especially guanosine/uridine (G/U)-rich single-stranded RNA, which engages endosomal TLR7/8 (Komura et al., 2020), and double-stranded RNA such as poly (I:C) (polyinosinic:polycytidylic acid) that triggers endosomal TLR3 (Lin et al., 2019) as well as cytosolic RIG-I (retinoic acid–inducible gene I) and MDA-5 (melanoma differentiation–associated antigen 5) (Bartok & Hartmann, 2020; Besch et al., 2009; Kato et al., 2008; Yoneyama et al., 2005) have also served as suitable molecular adjuvants in preclinical studies, especially when applied as nanoformulations, which on the one hand prevent degradation (Eygeris et al., 2022) and on the other hand augment the agonist's stimulatory activity as compared to application in soluble form (Tizard, 2021). These kinds of agonists were reported to enhance anti-tumour responses in clinical trials (Migliorini et al., 2019; Sun et al., 2022). In general, the co-delivery of adjuvants that engage distinct PAMP types may yield hyper-additive stimulatory effects as exemplified for CpG ODNs in combination with various other danger signals (Nigar & Shimosato, 2019).

As an alternative to nucleic acid-based sequences that trigger intracellular danger receptors, the DNA vaccine may comprise an additional expression unit that encodes for a signalling adaptor protein-like MyD88 (Collinson-Pautz et al., 2016) or a transcription factor like NF-κB [nuclear factor 'kappa-light-chain-enhancer' of activated B cells (Shedlock et al., 2014)] that act downstream of danger receptors to confer APC activation. In case that such minigenes are incorporated into the DNA vaccine, these may be transcriptionally regulated by a separate promoter (Mavi et al., 2019). Alternatively, such an expression unit may be linked to the antigen-coding sequence downstream of the stop codon by an internal ribosomal entry site (IRES) to confer cap-independent translation (Zhao, Sun, et al., 2023). Furthermore, both open reading frames may be fused and separated by a self-cleaving 2A peptide (Meas et al., 2021). By this approach, bicistronic and even multicistronic DNA vaccines can be generated (Shaimardanova et al., 2019).

All these adjuvants serve to achieve sustained immune cell activation, resulting in an upregulation of various effector proteins, comprising both transmembrane receptors such as MHC-I and MHC-II that present antigenic peptides to T cells and co-stimulatory receptors like CD86 as well as cytokines like IL-12 that promote the polarization of stimulated $CD4^+$ T cells towards Th1 (Mirlekar & Pylayeva-Gupta, 2021). However,

TABLE 3 Chemical and molecular adjuvants to enhance immunogenicity of DNA vaccines.

Adjuvant class	Example	Mechanism	References
Chemical adjuvants	Aluminium salt	Induction of cytokines, activation of complement system, induction of Th2 response	Eusébio et al. (2021); Grunwald & Ulbert (2015); Pagliari et al. (2023)
	Manganese	Elevation of metabolic immune cell activity (micronutrient), antioxidant defence, induction of cGAS-STING	Huang et al. (2023)
	NPs	Immunostimulatory potential (diverse mechanisms, including interactions with TLRs, complement activation, induction of autophagy); targeted and enhanced antigen delivery	Irvine et al. (2015); Liao et al. (2022)
	Oil-in-water emulsions (e.g. MF59)	Immunostimulatory potential, induction of cytokine production, depot effect	Eusébio et al. (2021); Hosseinipour et al. (2021); Ko and Kang (2018)
Molecular adjuvants (nucleic acids; proteins directly applied or DNA-encoded)	Poly(I:C)	TLR3, MDA-5 and RIG-I agonist; activation of NK cells, enhanced antigen presentation by DCs, induction of $CD8^+$ T-cell response	Bartok and Hartmann (2020); Besch et al. (2009); Eusébio et al. (2021); Kato et al. (2008); Lin et al. (2019); Yoneyama et al. (2005)
	CpG ODNs	TLR9 agonist; enhanced humoral immune response, induction of Th1 response and CTLs	Eusébio et al. (2021); Fehér (2019); Goonewardene et al. (2020); Kocabas et al. (2020); Xu et al. (2023)
	IL-12	Cytokine; induction of Th1 response	De Rosa et al. (2020); Jacobson et al. (2023); Mirlekar and Pylayeva-Gupta (2021)
	TNF-α	Cytokine; maturation and recruitment of immune cells	Nimal et al. (2006); Pagliari et al. (2023)
	IFN-γ	Cytokine; activation of immune cells (including T, B and NK cells), and other phagocytes, activation APCs, enhanced expression of MHC molecules	Pagliari et al. (2023); Tovey and Lallemand (2010)
	GM-CSF	Growth factor; recruiting of APCs, stimulation of DC maturation	Eusébio et al. (2021); Ruan et al. (2017)
	RANTES (CCL5)	Chemokine; activation of DCs and T cells	Cao et al. (2015); Eusébio et al. (2021)
	NF-κB	Transcription factor; regulation of cytokine expression, induction of DC maturation, activation of adaptive immune responses	Shedlock et al. (2014)
	MyD88	TLR signalling adaptor protein; induction of innate immune responses	Collinson-Pautz et al. (2016)
	CD40	Co-stimulatory receptor; induction of cytokine production by DCs, activation of B cells	Leng et al. (2022)
	shRNA, siRNA	RNA interference; immune induction by targeting of immunoinhibitory proteins	Almeida et al. (2015); Goel et al. (2024); Li & Petrovsky (2016); Setten et al. (2019)

Abbreviations: APC, antigen presenting cell; CCL, chemokine (C-C motif) ligand; CD, cluster of differentiation; cGAS, cyclic GMP-AMP synthase; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B cells; NK cells, natural killer cells; NPs, nanoparticles; ODNs, oligodeoxynucleotides; poly(I:C), polyinosinic:polycytidylic acid; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; shRNA, short hairpin RNA; siRNA, small interfering RNA; STING, stimulator of interferon genes; Th1, T helper cell type 1; Th2, T helper cell type 2; TLR, toll-like receptor; TNF-α, tumour necrosis factor-alpha.

numerous clinical trials have confirmed that vaccine immunogenicity may also be enhanced by forced over-expression of single effector proteins like IL-12 (De Rosa et al., 2020; Jacobson et al., 2023). Likewise, pre-clinical studies have shown that co-administration of a pDNA encoding for CD40 or CD63 can boost immune responses to a DNA vaccine against bovine viral diarrhoea virus in mice (Leng et al., 2022).

In a complementary approach, DNA vaccines may comprise a short hairpin (sh)RNA encoding cassette (Setten et al., 2019) that inhibits immuno-regulatory proteins. After processing, one strand of

shRNA-derived silencer (si)RNA will be incorporated into a RNA-induced silencing complex (RISC) that engages sequence-complementary mRNA stretches and either shortens half-life or attenuates translation of the target mRNA (Friedrich & Aigner, 2022). Interestingly, Almeida and co-workers showed that transfected pDNA triggered the STING pathway, which subsequently induced APOBEC (apolipoprotein B mRNA editing enzyme catalytic subunit) in an IFN-γ dependent manner (Almeida et al., 2015). APOBEC family members constitute single-stranded DNA cytosine deaminases that inhibit the replication of RNA and DNA viruses (Xu et al., 2020). APOBEC significantly reduced DNA transfection efficiencies, which was counteracted when using bicistronic vectors containing a APOBEC2-specific shRNA (Almeida et al., 2015). In order to promote immune responses, immunoinhibitory proteins may be targeted by shRNA. For example, the transcription factor STAT3 (signal transducer and activator of transcription 3) promotes tumorigenesis on the one hand by favouring tumour growth, establishment and maintenance of the tumour microenvironment (Dong et al., 2023), and on the other hand by imprinting a pro-tolerogenic state in APCs (Sohrabi et al., 2023). Co-delivery of tumour antigenic peptides, immunostimulatory CpG ODNs and STAT3-targeting shRNA synergistically enhanced anti-tumour responses in mice as compared to single treatment (Zhu et al., 2017). Furthermore, the efficacy of a pDNA encoding for GM-CSF and comprising an shRNA targeting furin that constitutes a pro-convertase of tolerance-promoting transforming growth factor beta protein boosted patients' anti-tumour immune responses in a number of clinical trials addressing distinct cancer types (Anderson et al., 2023; Barve et al., 2022; Ghisoli et al., 2015, 2016; Nemunaitis et al., 2014). In most preclinical and clinical studies, however, siRNA has been applied instead of shRNA, either alone or in combination with other agents to target pro-tumorigenic factors (Goel et al., 2024).

Finally, the mode of DNA vaccine delivery may affect and shape its adjuvancy as described above. For example, nanocarriers used for the delivery of DNA vaccines like liposomes (Gandhapudi et al., 2023), LNPs (Verbeke et al., 2022), PLGA NPs (Casey et al., 2019; Thirumalaikumar et al., 2023) and VLPs (Gupta et al., 2023; Pitoiset et al., 2017) may yield immunostimulatory activity. A broad overview of nanocarriers with adjuvant functions is given, for example, in Liao et al. (2022) and Zhang et al. (2024).

CONCLUDING REMARKS AND PERSPECTIVES FOR DNA VACCINE DESIGN

DNA vaccines have a broad therapeutic indication field, ranging from prevention of infections over treatment of infectious, allergic and autoimmune diseases to tumour immunotherapies (Eusébio et al., 2021). They offer several advantages as compared to mRNA-based vaccines, especially in terms of stability, transcriptional targeting and long-term expression (Hanke, 2022; Liu, 2019). Polyvalent plasmid design allows for co-expression of several antigens or antigen plus adjuvant, respectively (Kozak & Hu, 2024). Safety concerns such as genome integration or immunotoxicity have been refuted in many preclinical and clinical studies (Kozak & Hu, 2024). However, the efficacy of DNA vaccines in humans is still low and has to be increased for successful implementation in clinics (Pagliari et al., 2023). Poor immunogenicity as the major shortfall may be overcome by nanoformulated administration that enables coupled delivery of suitable adjuvants into the same target cell, specific APC targeting and efficient endosomal escape of the cargo after internalization (Ho et al., 2021; Lu et al., 2024; Mollé et al., 2022). Advances in NP engineering in the last years allow for tailor-made delivery systems with improved, specific physicochemical and biological properties. NPs can serve both as protective shuttles for the DNA vaccines and as immune stimulants. Furthermore, optimization of the DNA construct itself, including the deletion of prokaryotic sequences, employment of promoters with APC-focused activity and attachment of NLS to improve nuclear entry for enhanced expression may serve to increase transgene expression (Hager et al., 2020). Concerted implementation of these complementary optimization steps may increase the success of DNA vaccination in a synergistic manner. Also, alternative, topical administration routes (e.g. mucosal vaccines to enhance mucosal immunity as first barrier for pathogens) and innovative delivery devices (e.g. needle-free injection systems or inhalers), the combination with other (immuno)therapies, homologous or heterologous prime-boost strategies as well as optimized vaccination protocols (regarding application site, dose, frequency etc.) may enhance the clinical outcome of DNA vaccines (Lu et al., 2024; Zhang et al., 2015). A deeper understanding of the complex mechanisms of immunity is essential in terms of improved vaccine design and immunization/treatment regimes. Furthermore, interindividual differences in clinical responses have to be considered. In this regard, personalized medicine may be the solution, which can be realized by identification of individual (neo)antigens via bioinformatic and sequencing tools.

With view to the future, effective vaccination strategies are needed for existing and newly emerging infectious diseases (Gary & Weiner, 2020). DNA vaccines may provide here an optimal answer, especially when applied via innovative mucosal administration routes (e.g. intranasal, intratracheal or oral) since mucosal membranes are the first barrier for most of the

pathogens (Correa et al., 2022). Their cost-effective, easy, fast and scalable production allows for use in pandemics, where availability in short time, accessibility for a broad range of people (i.e. mass vaccination) and convenient application (i.e. painless and self-application/without special training) are pursued (Kozak & Hu, 2024; Lu et al., 2024). Yet, the storage issue (cold chain vs. room temperature, solid vs. liquid state) has to be addressed for worldwide use in different climate zones. Also, the field of tumour immunotherapies may benefit from DNA-based vaccines. Particularly, combinations with other approaches such as immune checkpoint inhibitors or chimeric antigen-receptor (CAR) T-cell therapies represent promising treatment strategies (Butterfield & Najjar, 2024; Lopes et al., 2019; Pandya et al., 2023).

All in all, the future of DNA vaccines looks encouraging. It remains exciting how this whole field will progress in the next years; further market approvals can be expected.

AUTHOR CONTRIBUTIONS

Simone Berger: Conceptualization; visualization; writing – original draft; writing – review and editing. **Yanira Zeyn:** Visualization; writing – original draft. **Ernst Wagner:** Conceptualization; writing – review and editing; funding acquisition. **Matthias Bros:** Conceptualization; funding acquisition; visualization; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest and no competing financial interest.

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