COMMENTARY

Building immune tolerance through DNA vaccination

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Duchenne muscular dystrophy (DMD) is a progressive, devastating disease of skeletal and cardiac muscles caused by loss of expression of the dystrophin protein (1). While the dystrophin gene and protein were discovered more than 30 y ago (2, 3), an effective treatment that can be administered to every patient, irrespective of their individual gene mutation, is still not available. Lessons and challenges about treating DMD have been learned over the years while testing various experimental therapies, including cell and gene replacement therapies (4, 5). One of the challenges faced by gene replacement techniques is the potential reaction of the immune system to a foreign protein. Indeed, previous reports have raised concerns over immune system reactions to transplanted donor cells (6), viral vector proteins, or dystrophin itself (7, 8). In the current study, Ho et al. (9) find that the immune system can be trained to accept foreign vectors and their encoded proteins through DNA vaccination (Fig. 1).

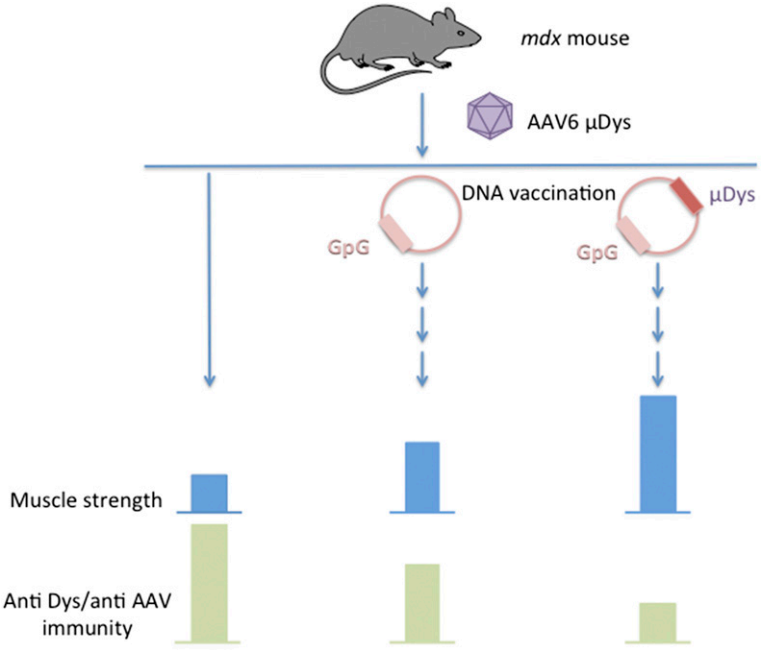

Fig. 1. Improving gene therapy outcomes through immune tolerization. An adeno-associated virus serotype 6 (AAV6) vector encoding a miniaturized version of dystrophin (mDys) was delivered to a mouse model of Duchenne muscular dystrophy (*mdx* mouse). The mice were subsequently treated weekly with an engineered plasmid in which all immunostimulatory CpG motifs had been replaced with immunosuppressive GpG motifs and encoded the same mDys gene. Control mice were treated with plasmid lacking the mDys gene or were injected with saline. Mice vaccinated with the engineered plasmid encoding the mDys gene showed improved muscle strength and reduced antibody-mediated immune responses to the dystrophin protein and AAV6 vector, relative to control animals.

Conceptually, gene therapy—the transfer of a "good" copy of a mutated or missing gene into a recipient cell—is the most direct way to achieve correction of many genetic disorders. It relies on delivering the therapeutic gene into the correct cell type, which for DMD is essentially all skeletal and cardiac tissue in the body. Although many different virus platforms have been investigated for delivery of the dystrophin gene in mouse models of DMD (including adenovirus, retrovirus, and lentivirus, among others), efficient body-wide transduction of the dystrophin gene has only been achieved using vectors based on adeno-associated virus (AAV) (10), with vector based on serotype 6 (AAV6) particularly good for muscle (11).

Unfortunately, AAV vectors have the capacity to hold only very small genes (~5 kbp in size), which creates additional challenges in the case of dystrophin, which is a relatively large gene with a minimum "full-length" size of about 11 kbp. Fortunately, the dystrophin protein has a modular construction, and the functionally crucial regions of the protein are mostly at the extreme ends of the protein, separated by a lengthy repeated region in the middle. Systematic structure/function analysis of the dystrophin protein identified miniaturized versions of the gene that encode a micro-dystrophin protein that retains almost full functionality yet is small enough to fit within the AAV capsid (12).

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While the human immune system provides surveillance to protect us from foreign invaders that seek to co-opt our cells and bodies for their own nefarious purposes (e.g., production and spread of progeny viruses or bacteria), it is incapable of distinguishing pathogenic viruses from beneficial gene therapy vectors. As such, delivery of all gene therapy vectors can elicit some degree of innate and/or adaptive immunity that can compromise therapy effectiveness and prevent vector readministration (13). This also extends to the therapeutic protein—a patient with DMD has never produced the dystrophin protein before, and dystrophin protein produced from a gene therapy vector can be viewed as "foreign" by the patient's immune system (8). Thus, immune responses to the vector and/or therapeutic protein can target the corrected cell for elimination by the patient's own immune system.

Identification of an effective gene delivery platform, AAV, and an appropriate therapeutic transgene, microdystrophin, allowed for development and testing of gene therapy approaches to treat DMD in preclinical trials in animal models of the disease and clinical trials in human patients (4). While AAV-microdystrophin proved very effective in inbred mouse models of the disease (11), immune responses to the therapeutic protein and gene therapy vector were observed in large animal models of DMD (e.g., golden retriever model of DMD) (14, 15) and in human patients (8). These studies clearly illustrate that generation of immune responses to foreign therapeutic proteins and/or gene therapy vectors is a real issue and can conspire to limit therapeutic efficacy.

The purpose of the study by Ho et al. was to test essentially a vaccination approach to achieve tolerization of the host immune system to the AAV-microdystrophin vector and therapeutic protein, using a mouse model of DMD, termed the mdx/mTR^{G2} mouse. One advantage to this approach is the ability to select the antigens to which the immune system should become tolerant, avoiding the need of a broad or nonspecific immunological suppression. Furthermore, the potential of developing selective immune tolerance to a foreign vector and its expressed transgene may open the possibility to administer serial therapeutic vector infusions. This is an important consideration for therapies of progressive diseases that do not directly correct the mutation present in the patient DNA but provide transgenic expression of the missing protein through a virus or other live carrier. Conversely, potential caveats of DNA vaccination in humans are the timing and duration of such vaccination regimen, in addition to its effectiveness at taming the immune system reactivity toward specific proteins. The present study administered the DNA vaccine for 32 consecutive weeks, the entire duration of the experiment, indicating that the proposed vaccine therapy might need to be maintained for the recipient lifetime.

Ho et al. also undertook a thorough evaluation of the possible immunogenic regions within microdystrophin and AAV6 that are prone to trigger an immune response. The data generated by these studies are informative and likely to have an impact toward engineering future AAV6-microdystrophin vectors to be used for gene therapy of *mdx*/DMD. For example, it appears that amino acids 286–305 of the AAV6 capsid protein may be a particularly strong immunological epitope. Similarly, some N- and C-terminal portions of human dystrophin induce a substantial antibody response, suggesting these are potentially strongly immunogenic regions of the protein. However, these same regions of dystrophin are also critical for function, with the N terminus linking to the actin cytoskeleton while the C terminus binds the dystroglycan and sarcoglycan complexes, thus forming a link to the extracellular matrix through the dystrophin–glycoprotein complex (16, 17). Given the critical function of these dystrophin domains, whether it is possible to remove additional portions of the protein, or alter the amino acid composition, to prevent immune reactivity without losing overall function and stability might be a future challenge.

There are two interesting side notes to the Ho et al. study. First, DNA vaccination did not necessarily lead to improved functional outcomes over what was achieved with the AAV-microdystrophin therapy alone. Although some muscle force measures were improved, there was no difference between the various treatment groups in cardiac function, number of dystrophin-positive muscle fibers, creatine kinase levels (a surrogate measure of muscle fiber damage), or the percentage of fibers with central nuclei (a marker of fiber regeneration). Similarly, the DNA vaccination regime did not significantly change T cell reactivity or the levels of circulating inflammatory cytokines in the animals. It may be that the AAV-microdystrophin therapy is so effective by itself that it is difficult to improve upon this, or that a greater effect may have been observed if this approach were investigated over a longer time frame. Second, the positive responses that were seen after vaccination with the microdystrophin-encoding plasmid (e.g., improved force generation and reduced antibody production) were also observed with the empty vector, albeit to a lesser extent. The backbone of the plasmid had been engineered to remove immunostimulatory CpG motifs (that can activate innate immune signaling through Toll-like receptor 9), which were replaced by immunosuppressive GpG motifs (18). The observation that the GpG-containing plasmid DNA can provide a generalized beneficial effect suggests that this nonspecific approach could be used to enhance the effectiveness, and reduce the immunogenicity, of many different gene therapy strategies for a variety of genetic diseases.

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An additional important observation from the present study is that portions of dystrophin not contained in the AAV6-microdystrophin vector trigger moderate immunogenicity, regardless of whether mice were treated with the DNA vaccine. These findings are in agreement with observations reported for DMD patients, suggesting that dystrophin produced by a small percentage of fibers, named "revertant" fibers, can be sufficient to trigger an immune response. This observation raises the question of whether starting DNA vaccination in *mdx* mice early in life, approximately at weaning age, could build better tolerance to foreign vectors. Indeed, revertant fibers have been shown as early as at 8 wk of age in *mdx* mice and they appear to significantly increase with age (19). Interestingly, a significant increase in revertant fibers was not seen in DMD patients in serial biopsies taken 8 y apart (20), suggesting there may be fundamental differences between human patients and mouse models of DMD or that increase in revertant fibers in DMD patients might occur over a longer time span. Nevertheless, spontaneous expression of foreign dystrophin from revertant fibers in the muscle of mice or humans is seen early in life, suggesting that preventive suppression of immunity toward dystrophin should be initiated shortly after birth.

Questions that remain to be addressed are whether DNA vaccination using dystrophin-expressing vectors could be used in place of immune suppressants, for how long the immune system will be tolerant toward dystrophin (re)expression, and whether its safety and sustainability are long-lasting. In theory, if true immune tolerance has been achieved, it should last a lifetime. However, we know that errors can occur and failed tolerance can build to autoimmunity. While some of these questions are still open, the present study offers an insightful and informative first glance at a problem that can make or break systemic delivery of foreign genes in models of genetic disorders. Biology can teach us lessons on how important it is to safely induce and maintain tolerance, for plenty of good causes.

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