

T Cell Responses to Dystrophin in a Natural History Study of Duchenne Muscular Dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is caused by the lack of dystrophin, but many patients have rare revertant fibres that express dystrophin. The skeletal muscle pathology of DMD patients includes immune cell infiltration and inflammatory cascades. There are several strategies to restore dystrophin in skeletal muscles of patients, including exon skipping and gene therapy. There is some evidence that dystrophin restoration leads to a reduction in immune cells, but dystrophin epitopes expressed in revertant fibres or following genome editing, cell therapy or microdystrophin delivery after AAV gene therapy may elicit T cell production in patients. This may affect the efficacy of the therapeutic intervention, and potentially lead to serious adverse events.

To confirm and extend previous studies, we performed annual Enzyme- Linked Immunospot interferon-gamma assays on peripheral blood mononuclear cells from 77 paediatric boys with DMD recruited into a natural history study, 69 of whom (89.6%) were treated with corticosteroids. T cell responses to dystrophin were quantified using a total of 368 peptides spanning the entire dystrophin protein, organized into nine peptide pools. Peptide mapping pools were used to further localize the immune response in one positive patient.

Six (7.8%) patients had a T cell-mediated immune response to dystrophin at at least one timepoint. All patients that had a positive result had been treated with corticosteroids, either prednisolone or prednisone.

Our results show that ~8% of DMD individuals in our cohort have a pre-existing T cell-mediated immune response to dystrophin despite steroid treatment. Although these responses are relatively low-level, this information should be considered as a useful immunological baseline before undertaking clinical trials and future DMD studies. We further highlight the importance for a robust, reproducible standard operating procedure for collecting, storing and shipping samples from multiple centres to minimise the number of inconclusive data.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive, progressive neuromuscular condition affecting 1:5000 male births¹. It is caused by mutations (mainly deletions) in the *DMD* gene, that codes for the protein dystrophin². The lack of dystrophin in skeletal muscle fibres causes them to degenerate; this is followed by cycles of regeneration/degeneration, ultimately resulting in the progressive loss of skeletal muscle (reviewed³). Individuals with DMD lose their ability to walk by their early teens, but corticosteroids can postpone the age at which ambulation is lost by 3–4 years⁴. Many approaches to restore dystrophin have been tested pre-clinically and four antisense drugs (eteplirsen, golodirsen, viltolarsen and casimersen) have been approved in the USA and a small molecule (ataluren) in Europe; these drugs restore a small amount of functional dystrophin. In addition, adeno-associated viral (AAV) gene therapy is a promising approach that is currently in clinical trials, e.g. ClinicalTrials.gov Identifiers: NCT04240314 AAV9, NCT02376816, NCT00428935 and NCT03368742 and EudraCT Number: 2020-002093-27.

There are several different dystrophin isoforms, but only the full-length dp427 is expressed in skeletal muscle fibres (reviewed^{1,2}). Dystrophin is not always completely absent in skeletal muscles of individuals with DMD: depending on the location of the mutation in the *DMD* gene, shorter, partially functional or non-functional dystrophin protein may be produced³. Many patients and mouse models have a small percentage of “revertant” muscle fibres that express truncated dystrophin protein⁵ (reviewed⁶). These revertant fibres arise from aberrant, stochastic splicing events that allow the production of small amounts of protein and the resulting dystrophin epitopes expressed in them⁷ might elicit T cell production. The latter may accelerate an immune response to the restored dystrophin in treated patients^{8,9} (reviewed¹⁰). Alternatively, dystrophin expressed in revertant fibres may reduce the immune response to myofibres expressing restored dystrophin¹¹. But the timing of the appearance of these revertant fibres is likely to be crucial. They are already present in newborn *mdx* mouse muscles⁷ and in DMD fetal muscle⁴ and their prenatal onset is likely to induce tolerance to the expressed epitopes.

It has been shown that increasing age correlates with an increased risk for a T cell-mediated immune response to dystrophin and in a previous cross-sectional study on 70 individuals with DMD, approximately 50% of the steroid naïve and 20% of the steroid treated population were reported to have circulating dystrophin primed T cells⁹. To confirm and extend these observations, we performed a multicentre, longitudinal natural history study, to determine whether patients with DMD had a pre-existing T cell-mediated immune response to dystrophin, and whether this changed over time. We performed Enzyme- Linked Immunospot (ELISPOT) IFN-gamma assays on individuals recruited into this four-year DMD natural history study that recruited 50 ambulant and 27 non-ambulant boys with DMD from four clinical centres. ELISPOT assays on all patients were performed with a full-length dystrophin peptide set; we also studied one individual who had an exon-skippable deletion with peptides corresponding to unique epitopes generated by the potential exon skipping event. We correlated our data to factors such as age, ambulation status, steroid regime and *DMD* deletion.

Methods

Subjects

Blood samples from DMD subjects belonging to a cohort of boys enrolled in the Association Francaise contre les Myopathies (AFM)-funded iMDEX multicentre natural history study were used for our experiments. Specimens from subjects recruited in London (Centre 1), Newcastle (Centre 2), Paris (Centre 3), and Leiden (Centre 4) were analyzed. All the samples used for this project are listed in Table 1.

This study was approved in the UK by the Bromley Research Ethics committee (REC 12/LO/0442), and the ethical committee of all the other institutions. All subjects and their legal representatives provided written informed consent for the study. This study is registered with the Clinical Trial Gov website with the number NCT02780492.

Boys with DMD were assessed annually over up to four years with an ELISPOT IFN- γ assay performed with a full-length dystrophin peptide set as previously described^{8,9}. A patient with an exon skippable deletion was additionally assessed with peptides corresponding to unique epitopes that would have been generated in the case of a single exon skipping

intervention to restore the reading frame. We also assessed four healthy adult controls as well as six neuromuscular disease controls from female children with non-dystrophinopathies. These consisted of one individual with muscle-eye-brain disease (6 years of age), four with limb girdle muscular dystrophy (9-16 years of age) and one with Ullrich congenital muscular dystrophy (14 years of age).

Sample collection and preparation

5-20 ml (ideally, at least 10 ml) of blood was collected from individuals into either heparin tubes, or Vacutainer® CPT™ Cell Preparation Tubes with sodium citrate. All samples were stored at room temperature for a maximum of 24 hours before processing. Samples from the two UK sites were delivered to UCL as blood within 24 hours and processed. Samples from France and the Netherlands were processed locally, and frozen peripheral blood mononuclear cells (PBMCs) shipped on dry ice to minimise loss of cell viability during shipment. A standardised procedure was used by all blood processing sites. Briefly, an equal volume of phosphate buffered saline (PBS) was added and a maximum of 20ml of diluted blood was carefully layered on top of 15 ml Ficoll. The tubes were centrifuged at 400g for 30 minutes at room temperature with slow acceleration and no brake. Plasma was removed, aliquoted and stored at -80°C; some of these samples were used for miRNA assays¹². The peripheral blood mononuclear cell (PBMC) layer was extracted and washed three times with 30ml PBS and centrifugation at 100g for 10 minutes at room temperature, with the brake on and high acceleration. The cell pellet was resuspended in chilled freezing medium (10% DMSO, 90% FCS) in 1ml aliquots (~10-20 million PBMCs/ml) and frozen in a Mr Frosty at -80°C overnight. When required, cells were thawed rapidly at 37°C, resuspended in 5 ml warmed AIM-V medium (AIM-V: Invitrogen, 12055-091) containing 2% human serum (Human AB serum, Gemini Bio Products 100-512, heat inactivated for 30 minutes at 56°C) and a count of viable cells was performed.

Peptides

20mer peptides overlapping by 10 amino acids that span the entire dystrophin protein were used (Proimmune Ltd, Oxford, UK). There was a total of 368 peptides, organized into 9 peptide pools⁸ (Figure 1). Stock vials of individual peptides at 5mg/ml were made up in 10% DMSO, 90% sterile water and stored at -80°C. Peptide pools were made up at 40

µg/ml (of each peptide), diluted in sterile water and kept at -80°C in small aliquots. Peptide mapping pools were used to further localize the immune response in a subset of positive individuals. In the mapping pools, each peptide is present in two sub-pools.

ELISPOT assay

Peripheral blood T cell responses to dystrophin were quantified using the ELISPOT assay⁸. This was performed using the Human IFN-gamma Elispot kit (U-CyTech, CT230-PB5) and Millipore IP filter plates (Millipore, S2EM004M99) according to the manufacturers' instructions.

3×10^5 cells were plated/well when screening for dystrophin responses and 75,000 cells/well were plated for the positive control or polyclonal stimulation. Concanavalin A (Sigma C0412) at final concentration of 2.5 µg/ml in PBS was used as a stimulus positive control. For peptide stimulations, the peptide pools were used at 1-2 µg/ml final concentration for each peptide. Cells were plated in duplicate wells, with a total volume of 200 µl (100 µl cells, 95 µl medium, 5 µl antigen). The ELISPOT plate was covered with a lid and incubated at 37°C, 5-7% CO₂ and 100% humidity for 24-36 hours.

Spots were imaged and counted using an automated AID reader. The same camera and count settings was used for all samples. Each well was manually assessed to remove any debris mistaken as spots. Over 50% saturation was considered too numerous to count. The intensity was set to a minimum of 20 brightness units and the spot size set as 40-500 pixels with a minimum gradient of 5 degrees. In line with Flanigan *et al*, a result was considered positive only when both duplicates were $> 15 \text{ SFC}/10^6 \text{ PBMCs}$ ⁸. We used this low threshold after discussion with Dr Mendell's group, whose reported responses to dystrophin are typically very low level⁸. For further confidence, in order to record a positive result, the positive control (patient's PBMCs reaction to Concanavalin A) must also be $> 15 \text{ SFC}/10^6 \text{ PBMCs}$ and the negative control (patient's PBMCs without stimulation) must be $< 5 \text{ SFC}/10^6 \text{ PBMCs}$.

Results

Boys with DMD were assessed annually (or semi-annually for patient 1.1) over three (and in one case four) years with an ELISPOT IFN-γ assay performed with a full-length

dystrophin peptide set⁹. The results are summarised in Figure 1 and Table 1 (full data in Supplementary Table S1). Twenty three patients from Centre 1, twenty patients from Centre 2, twenty three patients from Centre 3 and eleven patients from Centre 3 were included, ranging from 5-18 years of age. Details of the different steroid regimes are summarised in Supplementary Table S2. Thirty six were on prednisolone, sixteen were on deflazacort, fifteen were on prednisone, two were on prednisone followed by deflazacort. Of the participants that remained on the same treatment throughout, thirty nine were on a daily regime (fourteen prednisolone, fourteen deflazacort and eleven prednisone) and twenty seven were on an intermittent regime (twenty two prednisolone, two deflazacort and three prednisone).

A total of six (8%) individuals were positive at the first baseline visit (Figure 2). The positive epitopes are located before, and/or after the patient's deletion with no apparent associations (Supplementary Table 3). All individuals that had a positive result had been treated with corticosteroids (five with prednisolone and one with prednisone); two were ambulant and four were non-ambulant and ranged from 6-16 years of age (Supplementary Table 1). None of the sixteen deflazacort-treated patients had a positive result. We compared the rates of positive results between individuals that had been treated with prednisone, prednisolone, deflazacort, prednisone followed by deflazacort, compared to those on no/discontinued treatment and between individuals that had been treated with prednisolone compared to deflazacort, using a Fishers Exact Test. We found no significant differences between the any of the groups (deflazacort vs no/discontinued treatment - $p=1$; prednisone vs no/discontinued treatment - $p=1$; prednisolone vs no/discontinued treatment - $p=0.5661$; prednisone followed by deflazacort vs no/discontinued treatment - $p=0.2$; prednisolone vs deflazacort $P = 0.3077$).

The positive individuals had deletions in exons 45 (2 patients), 42-43, 48-50, 52 and 45-52 (Supplementary Tables 1 and 3). These mutations are expected to lead to the lack of full-length dystrophin (Dp427), but all should have been able to produce the shorter dystrophin proteins Dp116, Dp71 and Dp40, and one patient would be expected to produce Dp140 (Supplementary Table 3). None of these shorter dystrophin proteins, with the exception of Dp71¹³, are expressed in skeletal muscle¹⁴.

Due to the frequent occurrence of inconclusive results (defined below) at subsequent timepoints, we were unable to capture full longitudinal data on all individuals. Longitudinal data for two participants is presented in Figure 3. Patient 1.1 (patient 1 from Centre 1), carrying an exon 45 deletion, had an extremely strong response to peptide pool 7 (peptides encoded by exons 50-59) at baseline, which remained the strongest response among all pools at all subsequent visits (Figure 3). In contrast, patient 1.18, also deleted for exon 45, was only weakly positive for peptide pool 7 at baseline and 1-year follow-up but then showed a strong response to several peptide pools (including pool 7) at the 3-year visit (Figure 3).

Since patient 1.1 had a consistently strong response to peptide pool 7 at all timepoints, we performed additional ELISPOT assays using the mapping pool for peptide pool 7 at the zero and six-months timepoints (Figure 4). At the zero-year timepoint, this patient was positive for pools 7C, 7L, 7M and 7N, which together map to peptides encoded for by exon 54. The patient was also positive for pools 7A, 7L, 7M and 7N which maps to peptides encoded for by exon 51.

In summary, 4% of ambulant and 14.8% of non-ambulant patients had a T cell response to dystrophin. The mean ages of ambulant and non-ambulant patients at the start of the study were 8 and 14 years respectively. All the positive patients were taking prednisolone (five patients) or prednisone (one patient). Of the patients in our study taking prednisolone, 13.9% returned a positive ELISPOT result. Only eight patients had not been treated with corticosteroids and these either had a negative, or an inconclusive, result.

One patient (1.14, Supplementary table 1) with an exon 48-50 deletion, theoretically skippable for exon 51, was additionally assessed with peptides corresponding to unique junctional epitopes that would be generated by exon skipping. In this patient, the unique junctional epitope did not have a positive response, despite being positive to the full-length dystrophin peptide set (Figure 5). We also analysed four healthy adult controls and six disease controls which all returned a negative result (data not shown).

On the occasions when either the positive control was not positive, the negative control was positive and/or there were not enough viable cells to perform the assay samples were

scored as inconclusive (Supplementary table 1, marked with *). Sampling issues contributing to insufficient viable cells included the patient not attending the clinic, the appointment being cancelled, failure to take a blood sample, insufficient blood taken (a relatively high volume of blood (at least 10 ml) is required), or poor cell count and/or viability. There were (with rare exceptions) not enough cells remaining to perform repeat assays in cases where the original results were inconclusive. The assay was repeated on patient 3.1 at the first timepoint: the repeated assay was also inconclusive, as the negative control (patient's PBMCs without stimulation) was greater than 5 SFC/10⁶ PBMCs on both occasions.

Conclusions/Discussion

When designing clinical trials to restore dystrophin, it is important to identify patients that have a pre-existing T cell mediated immune response to dystrophin. It is also important to establish a natural history baseline and to try to understand factors that might affect this immune response and how it might be attenuated so that it does not interfere with treatment. This is especially important when considering AAV-mediated gene therapy approaches, which in pre-clinical work elicit significant dystrophin restoration (reviewed^{15,16,17}), although similar considerations apply to any experimental therapy employed to restore dystrophin, from genome editing to cell therapy. The fact that none of the neuromuscular disease controls had a T cell response to dystrophin suggests that a pathological muscle environment, including inflammation (reviewed¹⁸), does not on its own play an obvious role in the process. The muscle fibres of patients with other types of neuromuscular disease contain dystrophin, so they would have been tolerised to the protein. Our findings indicate that this tolerance was not broken by the immune cells, which would include T cells, that are present within pathological muscle. However, our small control group size is a limitation and a larger set of disease control individuals should be studied to conclusively address this question.

The ELISPOT assay is a highly sensitive and widely used immunoassay that measures the frequency of cytokine-secreting cells at the single-cell level (reviewed¹⁹). It is in theory easy to perform and provides both qualitative and quantitative information. Pre-existing cellular immune responses to dystrophin have already been reported and quantified using

the ELISPOT assay^{8,9}. The assay has also been used to examine T lymphocyte responses to dystrophin in a clinical trial of AAV-minidystrophin, showing that some patients had an immune response either before the start of treatment (2/6 patients), or after treatment (4/6 patients)⁹. These patients were given prednisolone 4 hours before treatment. In contrast, none of the 6 dogs included in a preclinical study of AAV-microdystrophin (that were transiently immunosuppressed with cyclosporine and mycophenolate mofetil had a post-treatment T cell response to dystrophin, but pre-treatment response was not quantified²⁰.

T cell-mediated immune responses to dystrophin in patients enrolled in our study were relatively rare and occurred at a fairly low level. Approximately 9% (6/69) of steroid treated DMD individuals had a pre-existing T cell-mediated immune response to dystrophin. A lower percentage of patients in our cohort had a response to dystrophin than in a previous study⁸, which reported that 20/70 (29%) of patients had T cell immunity against dystrophin. In this study, ninety-one subjects were enrolled, including 70 patients with DMD and 21 age-matched normal control subjects. Among the patients with DMD, 29 were treated with deflazacort, 24 were treated with prednisone and 17 were untreated. This may be due to the fact that the majority of our patients (69/77) were on corticosteroids, which would reduce the inflammatory response that occurs as part of the pathological process in dystrophin-deficient skeletal muscle (reviewed^{21,22}) and which may exacerbate any T cell response. In addition, the ages of the patients in the two studies were slightly different – from 4-18 years of age at the start of our study, and from 3-25 years of age in the Flanigan et al. study⁸. As older subjects were shown to have an increased probability of having an immune response to dystrophin⁸, it is possible that the different ages of the subjects in the two studies may have contributed to the different findings. But we cannot determine whether, as previously suggested⁸, a smaller percentage of patients that had been steroid treated compared to non-treated have an immune response to dystrophin, as we had so few patients (5/77) that were steroid-naïve (and none of these had a T cell response). The fact that all our positive patients were on prednisolone or prednisone is in accordance with Flanigan et al., who found a lower incidence of T cell response in patients treated with deflazacort than prednisolone⁸. But we found no significant differences in the percentage of individuals that had a T cell

response between those treated with either deflazacort or prednisolone. However, we only had sixteen deflazacort-treated patients in our study, which is too low to draw any firm conclusions.

Interestingly, the shorter dystrophin protein products not affected by the genomic deletions, which are therefore expected to be produced by the patients studied (Supplementary Table 2) did not appear to give any protection by tolerising against epitopes that they share with full-length dystrophin (Dp427). Revertant fibres, that are present in approximately 50% of individuals with DMD, might either tolerise the individuals, or induce an immune response to dystrophin. The fact that the number of revertant fibres does not change much with time⁵ and our findings that individuals often have a T cell response to dystrophin at one timepoint but not at others, argues against the idea that dystrophin in revertant fibres is eliciting the response.

Pre-existing immunity may be more of an issue in patients treated by gene therapy, which induces considerably higher levels of dystrophin production than exon skipping. In support of this, out of the 12 patients that had AON (Eteplirsen)-mediated restored dystrophin, there was no T cell response to dystrophin after 6 months of treatment⁸. Nevertheless, it is still important to identify, and if possible control, any immune response to dystrophin in patients both before they embark on any treatment intended to restore dystrophin and at timepoints after the onset of treatment.

Unfortunately, we encountered some issues that gave rise to inconclusive results in a high percentage of our assays (Supplementary Table 1). These included high background levels in negative controls that may be due to difficulties in processing blood within 24 hours. Twenty-three patients had an inconclusive result at their first timepoint. If these patients are removed from the analyses, then 11% of all patients had a positive result at at least one timepoint. Of all 185 assays performed, 74 (40%) had an inconclusive result. Eleven patients had either an inconclusive result, or sample problems at every timepoint. These problems may well have skewed our findings. To overcome such problems, we suggest that centre(s) collecting blood samples also isolate and freeze the PBMCs and send these, rather than the entire blood sample, to the laboratory doing the analysis. Obtaining a sufficient volume of blood (at least 10ml to achieve enough duplicate wells of 3×10^5

PBMCs/well) to isolate PBMCs can be challenging especially for younger DMD patients and those with neurobehavioural difficulties.

Despite the missing data indicated above, our longitudinal study clearly identified the T cell-mediated immune response to dystrophin in two DMD patients who were simply followed using standards of care. We complement and extend previous studies and show for the first time that having another type of muscular dystrophy, in which dystrophin is present, does not appear in itself to elicit an immune response to dystrophin. In line with the fact that an individual's immunological memory response can vary over time, we show that an individual's overall natural immune response to dystrophin, and response frequency can vary, as two patients that were positive at early timepoints were negative at the year 3 timepoint. Earlier work has suggested that the likelihood of an immune response to dystrophin increases with age⁵. In our cohort, four out of the six patients who has a positive response were above the mean age (9 years) at the start of the study.

Overall, it is likely that the responses we observed are a result of low avidity T cells that haven't quite escaped tolerance mechanisms. We cannot rule out cross-reactive responses from peptides that might be present in other proteins; it is also important to consider that each patient, even if they have the same deletion, will likely have different HLA types which might govern different responses. Our findings highlight the need for a robust, reproducible standard operating procedure for collecting, storing and shipping samples and for performing for assay, so that different intra and inter-laboratory operators achieve comparable results. Such a protocol could be used to routinely monitor patients' T cell response to dystrophin, especially in gene therapy clinical trials for DMD.

Further investigations of the T cell response might include use of the FluoroSpot assay, which utilizes fluorochrome-conjugated detection antibodies thereby allowing the simultaneous detection of several individual cytokines and subsequent analysis of T cell sub-populations^{23,24}. It would also be of interest to determine whether there is an anti-dystrophin humoral response in ELISPOT positive patients.

In conclusion, our results show that pre-existing T cell responses to dystrophin are uncommon (8%), inconsistent and low-level. Whilst this does provide some confidence for dystrophin restorative treatments, the fact that some patients are responsive warrants that

baseline T cell response should be considered before interpreting any data from dystrophin restoration.

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Author disclosure statement

Laurent Servais reports consultancy for Pfizer, Sarepta, Santhera, Biophytis, Regenx Bio. and is on the data and safety monitoring board of Fibrogen.

Imelda de Groot was in the past principal investigator of several drug trials (Italfarmaco, NSPharma, ReveraGen).

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Volker Straub is or has been on advisory boards for Astellas Gene Therapies, Biogen, Edgewise Therapeutics, Kate Therapeutics, ML Bio Solutions, Novartis Gene Therapies, Roche, Sanofi Genzyme, Sarepta Therapeutics, Vertex Pharmaceuticals and Wave

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Author contribution statement

KA: Methodology (equal), formal analysis (equal) Writing – original draft (supporting); Writing – review and editing (equal), Performed experiments (equal). PA: Methodology (equal), formal analysis (equal); Writing – review and editing (equal), Performed experiments (equal). FC: Methodology (equal), formal analysis (equal); Writing – review and editing (equal) Performed experiments (equal). JM: Methodology (equal), formal analysis (equal); Writing – review and editing (equal) Performed experiments (equal). JD: Methodology (equal), formal analysis (equal); MP: Methodology (equal), formal analysis (equal); Writing – review and editing (equal) Performed experiments (equal). VR: Methodology (equal), formal analysis (equal); Writing – review and editing (equal). KM: Writing – review and editing (equal). LCP: Writing – review and editing (equal). LS: Writing

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References

1. Mendell JR, Shilling C, Leslie ND, Flanigan KM, al-Dahhak R, Gastier-Foster J, et al. Evidence-based path to newborn screening for Duchenne muscular dystrophy. *Ann Neurol*. 2012;71(3):304-13.
2. Bladen CL, Salgado D, Monges S, Foncuberta ME, Kekou K, Kosma K, et al. The TREAT-NMD DMD Global Database: analysis of more than 7,000 Duchenne muscular dystrophy mutations. *Hum Mutat*. 2015;36(4):395-402.
3. Duan D, Goemans N, Takeda S, Mercuri E, Aartsma-Rus A. Duchenne muscular dystrophy. *Nat Rev Dis Primers*. 2021;7(1):13.
4. Ricotti V, Ridout DA, Pane M, Main M, Mayhew A, Mercuri E, et al. The NorthStar Ambulatory Assessment in Duchenne muscular dystrophy: considerations for the design of clinical trials. *J Neurol Neurosurg Psychiatry*. 2016;87(2):149-55.
5. Arechavala-Gomez V, Kinali M, Feng L, Guglieri M, Edge G, Main M, et al. Revertant fibres and dystrophin traces in Duchenne muscular dystrophy: implication for clinical trials. *Neuromuscul Disord*. 2010;20(5):295-301.
6. Lu QL. Revertant Phenomenon in DMD and LGMD2I and Its Therapeutic Implications: A Review of Study Under Mentorship of Terrence Partridge. *J Neuromuscul Dis*. 2021;8(s2):S359-S67.
7. Lu QL, Morris GE, Wilton SD, Ly T, Artem'yeva OV, Strong P, et al. Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J Cell Biol*. 2000;148(5):985-96.
8. Flanigan KM, Campbell K, Viollet L, Wang W, Gomez AM, Walker CM, et al. Anti-dystrophin T cell responses in Duchenne muscular dystrophy: prevalence and a glucocorticoid treatment effect. *Hum Gene Ther*. 2013;24(9):797-806.
9. Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, et al. Dystrophin immunity in Duchenne's muscular dystrophy. *N Engl J Med*. 2010;363(15):1429-37.

10. Moore MJ, Flotte TR. Autoimmunity in a genetic disease—a cautionary tale. *N Engl J Med*. 2010;363(15):1473-5.
11. Ferrer A, Wells KE, Wells DJ. Immune responses to dystropin: implications for gene therapy of Duchenne muscular dystrophy. *Gene Ther*. 2000;7(17):1439-46.
12. Catapano F, Scaglioni D, Maresh K, Ala P, Domingos J, Selby V, et al. Novel free-circulating and extracellular vesicle-derived miRNAs dysregulated in Duchenne muscular dystrophy. *Epigenomics*. 2020;12(21):1899-915.
13. Kawaguchi T, Niba ETE, Rani AQM, Onishi Y, Koizumi M, Awano H, et al. Detection of Dystrophin Dp71 in Human Skeletal Muscle Using an Automated Capillary Western Assay System. *Int J Mol Sci*. 2018;19(6).
14. Muntoni F, Torelli S, Ferlini A. Dystrophin and mutations: one gene, several proteins, multiple phenotypes. *Lancet Neurol*. 2003;2(12):731-40.
15. Duan D. Micro-Dystrophin Gene Therapy Goes Systemic in Duchenne Muscular Dystrophy Patients. *Hum Gene Ther*. 2018;29(7):733-6.
16. Crudele JM, Chamberlain JS. AAV-based gene therapies for the muscular dystrophies. *Hum Mol Genet*. 2019;28(R1):R102-R7.
17. Asher DR, Thapa K, Dharia SD, Khan N, Potter RA, Rodino-Klapac LR, et al. Clinical development on the frontier: gene therapy for duchenne muscular dystrophy. *Expert Opin Biol Ther*. 2020;20(3):263-74.
18. Tidball JG, Welc SS, Wehling-Henricks M. Immunobiology of Inherited Muscular Dystrophies. *Compr Physiol*. 2018;8(4):1313-56.
19. Yang F, Patton K, Kasprzyk T, Long B, Gupta S, Zoog SJ, et al. Validation of an IFN-gamma ELISpot assay to measure cellular immune responses against viral antigens in non-human primates. *Gene Ther*. 2021.
20. Shin JH, Pan X, Hakim CH, Yang HT, Yue Y, Zhang K, et al. Microdystrophin ameliorates muscular dystrophy in the canine model of duchenne muscular dystrophy. *Mol Ther*. 2013;21(4):750-7.

21. Quattrocelli M, Zelikovich AS, Salamone IM, Fischer JA, McNally EM. Mechanisms and Clinical Applications of Glucocorticoid Steroids in Muscular Dystrophy. *J Neuromuscul Dis.* 2021;8(1):39-52.
22. Kourakis S, Timpani CA, Campelj DG, Hafner P, Gueven N, Fischer D, et al. Standard of care versus new-wave corticosteroids in the treatment of Duchenne muscular dystrophy: Can we do better? *Orphanet J Rare Dis.* 2021;16(1):117.
23. Janetzki S. Immune monitoring technology primer: the enzyme-linked immunospot (Elispot) and Fluorospot assay. *J Immunother Cancer.* 2015;3:30.
24. Korber N, Behrends U, Hapfelmeier A, Protzer U, Bauer T. Validation of an IFN γ /IL2 FluoroSpot assay for clinical trial monitoring. *J Transl Med.* 2016;14(1):175.

Figure legends

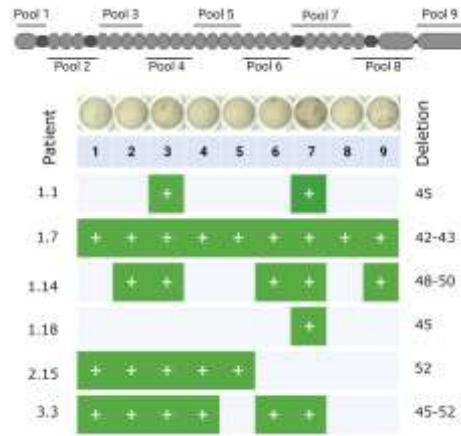


Figure 1.

The location of the nine dystrophin peptide pools are illustrated in relation to the structural features of the dystrophin protein. An example of ELISPOT wells from one patient (Centre 1, patient 14; (Supplementary Table 1) at the 0-year timepoint is provided. The table summarises the results of the six positive patients at the 0-year timepoint showing which peptide pools returned a positive result.

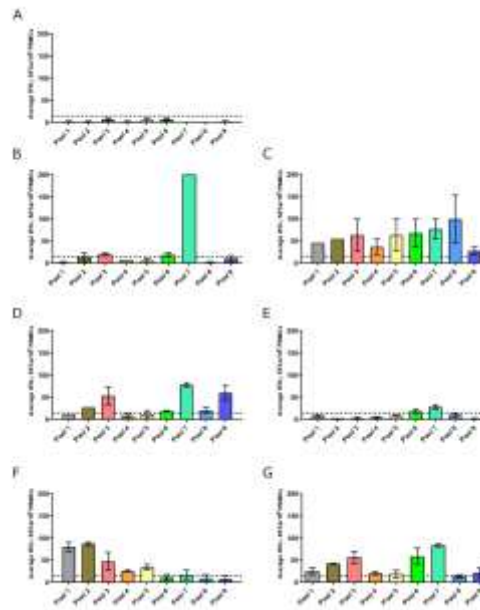


Figure 2.

Graphs plotting the average (\pm SEM) IFN- γ SFCs/ 10^6 PBMCs values across each peptide pool for: A) a representative example of a negative sample (patient 1.11 at the 0-year timepoint); B) positive patient 1.1; C) positive patient 1.7; D) positive patient 1.14; E) positive patient 1.18; F) positive patient 2.15 and G) positive patient 3.3. The dotted line represents the positive cut-off value of 15 SFCs/ 10^6 PBMCs; note both duplicates must be >15 to be considered positive. Where a bar reaches 200 SFCs/ 10^6 PBMCs, the spots were too numerous to count.

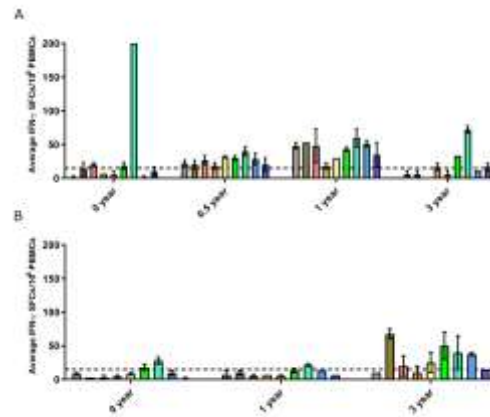


Figure 3.

ELISPOT results for patients 1.1 (A) and 1.18 (B) over time. The graphs plot the average (\pm SEM) IFN- γ SFCs/ 10^6 PBMCs values across each peptide pool. The dotted line represents the positive cut-off value of 15 SFCs/ 10^6 PBMCs; note both duplicates must be >15 to be considered positive. Where a bar reaches 200 SFCs/ 10^6 PBMCs, the spots were too numerous to count.

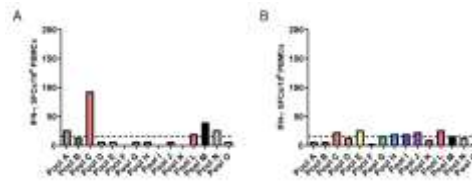


Figure 4.

Results from mapping pool 7 at zero (A) and 6-months (B) timepoints (patient 1.1). The graphs plot the IFN- γ SFCs/10⁶ PBMCs values across each mapping pool. The dotted line represents the positive cut-off value of 15 SFCs/10⁶ PBMCs; note both duplicates must be >15 to be considered positive.

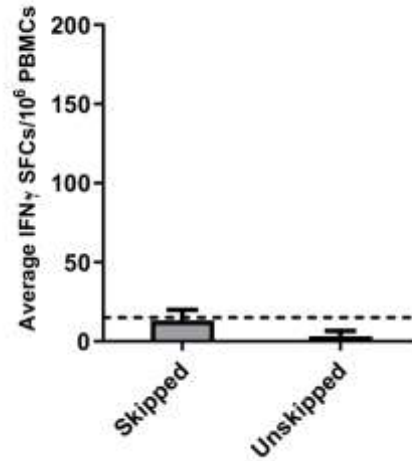


Figure 5.

Graph plotting the average (\pm SEM) IFN- γ SFCs/10⁶ PBMCs values for a peptide pool corresponding to the unique epitopes that would be generated by exon 51 skipping for patient 1.14 (48-50 deletion). A peptide pool for the unskipped scenario was also tested. The dotted line represents the positive cut-off value of 15 SFCs/10⁶ PBMCs; note both duplicates must be >15 to be considered positive.

Table 1. Summary of elispot results

Cohort	Total	No. positive	% positive
Control (healthy)	5	0	0
Control (disease)	6	0	0
DMD total	77	6	7.8
DMD ambulant	50	2	4
DMD non-ambulant	27	4	14.8
DMD no/discontinued steroids	8	0	0
DMD deflazacort	16	0	0
DMD prednisone	15		0
DMD prednisolone	36	5	13.9
DMD prednisone followed by deflazacort	2	1	50