Strategies for the Induction of Immune Tolerance to Enzyme Replacement Therapy in Mucopolysaccharidosis Type I

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Enzyme replacement therapy with laronidase is an established treatment for Mucopolysaccharidosis type I (MPS I), but its efficacy may be limited by the development of anti-drug antibodies, which inhibit cellular uptake of the enzyme. In a related disorder, infantile Pompe disease, immune tolerance induction with low-dose, short-course methotrexate appears to reduce antibody formation. We investigated a similar regimen using oral methotrexate in three MPS I patients. All patients developed anti-laronidase immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies, and they had clinically relevant levels of cellular uptake inhibition. We then explored several immune tolerance induction strategies in MPS I mice: (1) methotrexate, (2) combination of non-depleting anti-CD4 and anti-CD8 monoclonal antibodies, (3) methotrexate with anti-CD4 and anti-CD8 monoclonals, (4) anti-CD4 monoclonal, and (5) anti-CD8 monoclonal. Treated mice received 10 weekly laronidase injections, and laronidase was delivered with adjuvant on day 49 to further challenge the immune system. Most regimens were only partially effective at reducing antibody responses, but two courses of non-depleting anti-CD4 monoclonal antibody (mAb) ablated immune responses to laronidase in seven of eight MPS I mice (87.5%), even after adjuvant stimulation. Immune tolerance induction with methotrexate does not appear to be effective in MPS I patients, but use of non-depleting anti-CD4 monoclonal is a promising strategy.

INTRODUCTION

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive lysosomal storage disorder (LSD) caused by the deficiency of α-L-iduronidase (IDUA), resulting in the lysosomal accumulation of the glycosaminoglycans (GAGs) heparan sulfate (HS) and dermatan sulfate (DS). This leads to progressive multisystem and organ dysfunction.[1] There is a continuous phenotypic spectrum from severe to attenuated disease. In the most severe form (Hurler syndrome [MPS IH]), there is early and progressive CNS involvement in addition to somatic features, such as skeletal dysplasia, hepatosplenomegaly, dysmorphism, and respiratory and cardiac involvement. At the most attenuated end of the spectrum, there may be normal life expectancy and no CNS involvement. The two therapeutic strategies available for MPS I patients are hematopoietic stem cell transplant (HSCT) and intravenous enzyme replacement therapy (ERT).

ERT is the primary treatment option for individuals with attenuated phenotypes, but it does not cross the blood-brain barrier and, therefore, cannot treat the CNS manifestations of the severe form of the disease (MPS IH). HSCT allows donor-derived cells to engraft in the CNS and secrete enzyme, cross-correcting neighboring cells. HSCT is therefore the treatment of choice for MPS IH, and it can prevent neurological decline if performed early in the disease course.[2] Many clinical centers also give ERT to patients in conjunction with HSCT, in order to reduce substrate storage levels and improve the clinical condition of the patient prior to transplantation,[3] and, in selected patients, improvement in cardio-respiratory function may help them better tolerate the intensive conditioning regimen.[4,5]

The formation of anti-drug antibodies to ERT has been reported in all LSDs treated with ERT.[6,7] In MPS I, the formation of functional antibodies against ERT has been widely reported in MPS I animals as well as patients. MPS I dogs that had little or no antibody responses against ERT showed higher enzyme level, improved GAG reduction in organs including the brain, and better improvement in lysosomal pathology compared to those with higher antibody responses.[8,9] In humans, over 80% of MPS I patients develop high antibody titers to ERT, with 35% of these incidences being associated with significant antibody-mediated inhibition of enzyme uptake into cells.[10]

While patients without antibody responses demonstrated biochemical and clinical improvements that were comparable to patients

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Table 1. Participants Recruited to the Immune Tolerance Induction with Methotrexate in Hurler Syndrome Study

Subject	Sex	Age at Enrollment (Months)	Genotype	Iduronidase Enzyme Activity (umol/g/h) (Reference Range 10–50)	Number of Weekly Doses of ERT Pre-HSCT	Intervention
1	male	4.5	c.1205G > A/c.1205 G > A [p.(Trp402Ter)/p.(Trp402Ter)/]	0.02	9	1 cycle methotrexate
2	female	11.5	c.1205G > A/c.979G > C [p.(Trp402Ter)/p.(Ala327Pro)]	0.17	12	1 cycle methotrexate
3	male	13.6	c.1205G > A/c.46_57del12 [p.(Trp402Ter) /p.(Ser16_Ala19del)]	undetectable	10	3 cycles methotrexate

Participants were between 4 and 13 months of age at enrollment, and they received between 9 and 12 doses of ERT pre-HSCT.

receiving HSCT, a high inhibitory antibody level was found to correlate with poorer biomarker outcomes (e.g., GAG levels, DS:chondroitin sulfate [CS] ratio, and heparin cofactor II thrombin complex [HCII-T]) as well as the occurrence of severe sleep-disordered breathing.[10–12] Specifically, uptake inhibition of >30% with total antibody titers of >1:4,000 was correlated with the occurrence of severe sleep-disordered breathing, and these could be considered thresholds for a clinically relevant antibody response.[12] The development of high levels of uptake inhibitory antibodies has also been implicated in suboptimal response to therapy in a study of long-term outcomes of UK patients with attenuated MPS.[13]

An effective immune tolerance induction (ITI) regimen is, therefore, much needed in order to improve the treatment outcome for MPS I patients. To date, pharmacological measures to prevent antibody responses in MPS I patients have not been successful.[14] HSCT remains the only effective strategy to either completely abolish or reduce antibody responses to a clinically insignificant level in MPS I patients, though antibody responses may persist for several months post-transplant and are associated with an arrest in biomarker responses, suggesting cross-reactivity between antibodies and donor-derived enzyme.[11]

An immune tolerance induction regimen based on methotrexate (MTX), rituximab, and intravenous immunoglobulin has been successfully used to prevent the development of antibodies to ERT with alglucosidase alfa in patients with infantile Pompe disease,[15,16] a condition in which the development of high-titer antibodies is associated with significant morbidity and mortality.[17] Methotrexate is a dihydrofolate reductase antagonist that induces immune tolerance by provoking the death of proliferating T and B cells, via inhibiting purine and pyrimidine metabolism and DNA *de novo* synthesis.[18] Low-dose, short-course methotrexate monotherapy was shown to prevent antibody responses to recombinant human α-glucosidase in a Pompe mouse model,[19,20] with early data suggesting this strategy may also be effective in patients.[21,22] The efficacy of such a regimen was, therefore, investigated in patients with MPS IH.

A variety of immune tolerance induction strategies was also explored in an MPS I mouse model. The roles of regulatory T cells in the regulation of allergen-specific immune response and autoimmunity have been documented previously. They generate immune tolerance by releasing suppressor cytokines interleukin-10 (IL-10) and transforming growth factor β (TGF-β), which play a role in suppressing immunoglobulin E (IgE) production, and inhibiting T cell and B cell generation.[23] In this study, we evaluated the efficacy of immune tolerance induction for these regimens: a combination of non-depleting anti-CD4 and anti-CD8 monoclonal antibodies (mAbs), methotrexate, and methotrexate with anti-CD4 and anti-CD8 monoclonal antibodies. Non-depleting anti-CD4 and anti-CD8 monoclonal antibodies have been reported to induce regulatory T cell production and promote long-term acceptance in allogenic transplant.[24,25] Methotrexate was able to induce immune tolerance to recombinant human acid α-glucosidase in a Pompe mouse model.[20] These regimens were evaluated for their efficacy to induce immune tolerance to laronidase using an MPS I mouse model.

RESULTS
Low-Dose, Short-Course Oral Methotrexate Monotherapy in MPS IH Patients
Methotrexate Is Well Tolerated and Not Associated with Significant Adverse Effects

An immune tolerance induction regimen based on oral methotrexate was investigated in three individuals with MPS IH (Table 1). Treatment-emergent adverse events (TEAEs) are summarized in Table 2. Most TEAEs were due to intercurrent viral infections or infusion reactions to laronidase infusion, the latter occurring in subjects 1 and 3. The two serious adverse events were both for subject 3 and were related to the underlying condition of MPS I (planned hospital admission for hip arthroscopy; admission to commence ACE inhibitors for mitral regurgitation), but not related to the study intervention. No other serious TEAEs were reported. Only two methotrexate-related TEAEs were reported, neither of which were serious TEAEs. Subject 1 developed mild elevations of liver transaminases (<3× upper limit of normal = grade 1, Common Terminology Criteria for Adverse Events [CTCAE], version 3.0), which were judged to be probably related to methotrexate administration. Alanine aminotransferase (ALT) was elevated at week 1 and resolved by week 2. Aspartate aminotransferase (AST) was elevated at week 2 and resolved by week 3. Participant 2 developed thinning of hair and mild (CTCAE grade 1) hair loss at week 5, which was judged to be possibly related to methotrexate and resolved without intervention by week 9.

Table 2. Treatment-Emergent Adverse Events in the Immune Tolerance Induction with Methotrexate in Hurler Syndrome Study

Event	Number of Participants (Number of Events)
Any TEAE	3 (15)
Methotrexate-related TEAEs	2 (2)
Serious TEAEs	1 (2)
Methotrexate-related serious TEAEs	0 (0)
Interruption or discontinuation of methotrexate regimen due to TEAE	0
Discontinuation from study due to TEAE	0

No participants experienced treatment-related serious adverse events, and no participants withdrew from the study due to adverse events.

Anti-laronidase IgG Antibodies, IgM Antibodies, and Cellular Uptake Inhibition in MPS I Patients Treated with Methotrexate

Treatment with methotrexate did not lead to a clinically relevant reduction in anti-laronidase immunoglobulin G (IgG) titers. Both participants who received 1 cycle of methotrexate developed high-titer anti-laronidase IgG antibodies. A third participant received 3 cycles of methotrexate (Figure 1A) but also developed high-titer anti-laronidase IgG antibodies (Figure 1B). All participants developed antibody titers above 1:4,000, which are considered clinically relevant.[12] On the basis of these results, the study was terminated and no further participants were recruited. An antibody response was evident by week 4 in all participants, and a relative reduction in antibody titers post-HSCT was observed in all participants. The peak anti-laronidase IgG antibody titer between commencing ERT and HSCT was comparable to that seen in a historical group of MPS IH patients who did not receive immune tolerance induction[11] (Figure 1C).

Inhibition of cellular uptake due to antibody-mediated inhibition was observed in all patients, suggesting potential functional relevance of the antibody response (Figure 1E).

An anti-laronidase immunoglobulin M (IgM) antibody response was evident at the week 4 time point in all participants, and it rapidly decreased at subsequent time points (Figure 1D). For subject 3, the week 4 time point was <7 days after administration of the final dose of methotrexate.

Biomarker Response to Laronidase and Regulatory T Cell Populations

Biomarker reduction was evident after the commencement of ERT, as previously described in MPS IH patients receiving pre-transplant ERT, but differences only became statistically significant at the post-HSCT time point (Figures 2A–2C). Total urine GAG decreased from a mean of 241.6 mg/mmol creatinine at baseline to 96.8 mg/mmol creatinine pre-HSCT, and it further decreased to a mean of 28.8 mg/mmol creatinine post-HSCT (p = 0.0195) (Figure 2A). Urine HS + DS decreased from a mean of 72,980 μg/mmol creatinine at

baseline to 25,831 μg/mmol creatinine at week 8 (Figure 2B). At least one value was missing at later time points. The differences were not statistically significant in repeated-measures analysis. Serum HS + DS decreased from a mean of 3,161 ng/mL at baseline to 906 ng/mL pre-HSCT, and it further decreased to 631 ng/mL post-HSCT (p = 0.0298) (Figure 2C).

No increase in regulatory T cell population from baseline was seen in any participant. Differences were not statistically significant in repeated-measures analysis (Figure 2D; Figures S1 and S2).

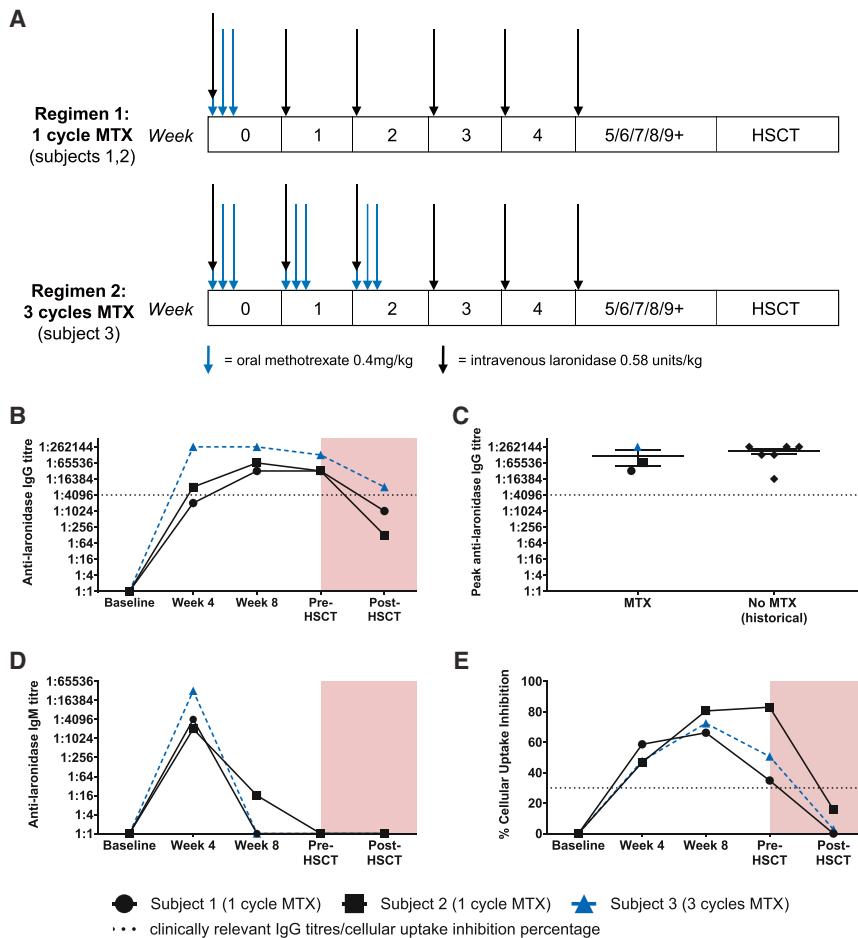### Immune Tolerance Induction Strategies in an MPS I Mouse Model

Methotrexate Reduces Antibody Responses to ERT in MPS I Mice in the Short Term

To re-optimize the effective dose for methotrexate that can induce immune tolerance to ERT in MPS I mice, a range of methotrexate doses with different delivery schedules was tested on MPS I mice (Figure 3A). All MPS I mice in the ERT group developed significant laronidase-specific antibodies by day 28 following 4 weekly administrations of laronidase (Figure 3B). All methotrexate-treated groups developed lower average antibody responses than the positive control group. Antibody levels in MPS I mice that received three cycles of 0.5 mg/kg methotrexate, one cycle of 1.6 mg/kg methotrexate, one cycle of 5 mg/kg methotrexate, and one course of 16 mg/kg methotrexate were significantly lower than the ERT group (p = 0.0018, p = 0.00109, p = 0.0008, and p = 0.0019, respectively). The antibody levels in these three treated groups on day 28 were 3.91-fold, 5-fold, and 3.89-fold lower than the ERT group, respectively. As a result, the minimum effective dose of one cycle of 5 mg/kg methotrexate was brought forward to use in the following experiment.

Combined Treatment with Anti-CD4 and Anti-CD8 Monoclonal Antibodies, but Not Methotrexate, Reduces Antibody Responses to ERT in the Longer Term

The abilities to reduce anti-laronidase IgG antibodies in MPS I mice with a single cycle of methotrexate compared with either anti-CD4 and anti-CD8 non-depleting monoclonal antibodies or a combined treatment group were investigated (Figure 4A). All MPS I mice that received ERT developed anti-laronidase IgG antibodies over the 70-day treatment period. On day 28, ERT-treated mice showed significant anti-laronidase responses (p = 0.0103). The average anti-laronidase IgG antibody levels in the methotrexate group, monoclonal antibodies group, and the combined group were 1.7-fold, 9-fold, and 2.2-fold lower than the ERT group, respectively, but only the monoclonal antibodies group was significantly lower (p = 0.0092).

Adjuvant stimulation was performed at day 49 to exacerbate potential anti-ERT responses, given the robust immune responses observed in patients. After adjuvant stimulation at day 70, ERT-treated mice had further elevated anti-laronidase IgG antibody levels (p < 0.0001). Methotrexate was unable to reduce antibody responses in MPS I mice (ERT group, 31.7 μg/mL; methotrexate group, 33.8 μg/mL).

Figure 1. Immune Tolerance Induction with Methotrexate in Hurler Syndrome Study Schedule and Antibody Responses to Laronidase in MPS I Hurler Patients

(A) Immune tolerance induction with methotrexate in Hurler syndrome study schedule. The first two participants were allocated to regimen 1 (1 cycle of methotrexate). A subsequent participant was allocated to regimen 2 (3 cycles of methotrexate). Each cycle comprised three doses of 0.4 mg/kg methotrexate administered orally around an infusion of laronidase (60 min prior to infusion, 24 h after infusion, and 48 h after infusion). (B) All participants developed high titers of IgG to laronidase despite the methotrexate regimen. An antibody response was evident by week 4, and a relative reduction in antibody titers was evident post-HSCT. (C) Peak anti-laronidase IgG titers were comparable to those seen in a historical longitudinal study[11] of antibody responses to laronidase. (D) An IgM response was evident by week 4 in all participants, including subject 3 who received methotrexate for 3 cycles (week 4 time point was <7 days following the final dose of methotrexate). (E) All participants developed evidence of antibody-mediated inhibition of cellular uptake of enzyme. Dotted line in (B) and (C) represents clinically relevant anti-laronidase IgG titers (1:4,096); dotted line in (E) represents clinically relevant cellular uptake inhibition of percentage (<30%).

the adjuvant stimulation, anti-laronidase IgG antibody levels were further elevated in ERT-treated MPS I mice. Only anti-CD4 monoclonal antibody-treated mice had significantly lower anti-laronidase IgG antibody levels than positive controls (p < 0.0001). Indeed, seven of eight MPS I mice (87.5%) in the anti-CD4 monoclonal antibody-treated group showed very low or completely absent antibody responses. In contrast, anti-CD8 monoclonal antibody was unable to reduce antibody responses to laronidase after adjuvant stimulation (Figure 5B).

Only Two Cycles of Anti-CD4 Monoclonal Antibodies Are Able to Completely Eradicate Antibodies Inhibiting the Cellular Uptake of Laronidase

Antibody-mediated cellular uptake inhibition was observed in all treated mice except those receiving 2 cycles of anti-CD4 monoclonal antibodies. Mice from the ERT-only group showed 70% of cellular uptake inhibition. Though all immune tolerance induction treated groups showed significantly less cellular uptake inhibition (methotrexate group, p = 0.0447; monoclonal antibody group, p = 0.0001; methotrexate + monoclonal antibodies group, p = 0.0001; anti-CD4 monoclonal antibody group, p = 0.0001; and anti-CD8 monoclonal antibody group, p = 0.0221), the percentage of uptake inhibition in the methotrexate- (44.9%) and anti-CD8 monoclonal antibody-treated groups (41.5%) remained clinically relevant (>30%). On the other hand, mice that received monoclonal antibodies (23.8%) or monoclonal antibodies + methotrexate (11.3%) (both containing anti-CD4 monoclonal antibodies) only developed low levels of
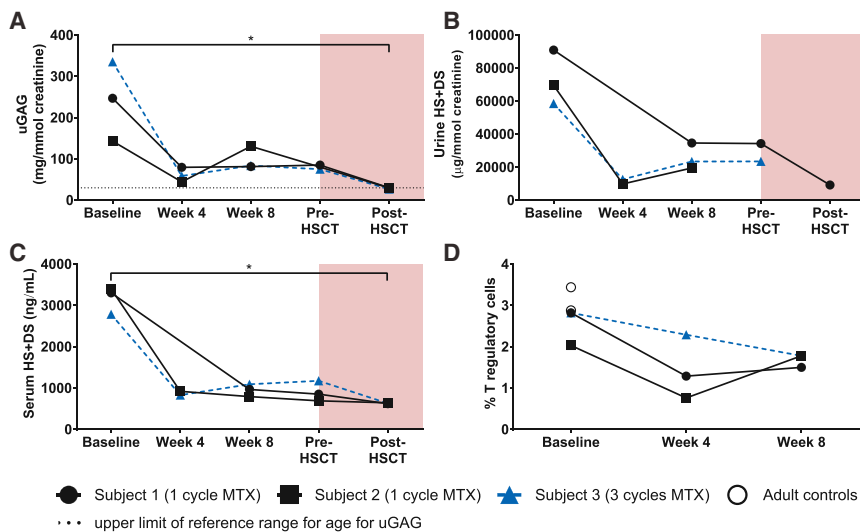
Both MPS I mice groups that received monoclonal antibodies (monoclonal antibodies alone and a combination of methotrexate and monoclonal antibodies) showed significantly lower anti-laronidase IgG antibody levels on day 70 (monoclonal antibodies group, 13.5 μg/mL, p < 0.0001; methotrexate + monoclonal antibodies group, 17.4 μg/mL, p = 0.0001) (Figure 4B), although neither treatment normalized antibody responses.

Two Cycles of Anti-CD4 Monoclonal Antibody Counteract the Anti-laronidase Antibody Responses Raised by the Adjuvant

In an attempt to further reduce immune tolerance induction responses observed in the anti-CD4/anti-CD8 group and determine the importance of CD4 or CD8 cells in the response, MPS I mice received two cycles of either anti-CD4 monoclonal antibody or anti-CD8 monoclonal antibody when they were given the adjuvant stimulation on day 49 (Figure 5A). On day 28, ERT-treated MPS I mice showed a significant elevation of anti-laronidase IgG antibody levels. Mice that received anti-CD4 monoclonal antibody were 11.4-fold lower than the ERT-only group (p = 0.0164) and not significantly different from the untreated control. Mice that received anti-CD8 monoclonal antibody were neither significantly less than ERT treated nor more than negative controls. On day 70, 21 days after

Figure 2. Biomarker Responses to Laronidase in MPS I Hurler Patients Receiving Immune Tolerance Induction with Methotrexate

(A) Total urinary glycosaminoglycan in all patients. (B) Heparan sulfate and dermatan sulfate concentrations in urine in all patients. (C) Heparan sulfate and dermatan sulfate concentrations in serum in all patients. (D) Treg population in patient PBMCs, expressed as the percentage of $CD4^+$ T cells. Individual data points are shown; *$p < 0.05$. Dotted line in (A) represents the upper limit of reference range for age.

methotrexate. No evidence of an immunosuppressive biological effect of methotrexate on regulatory T cell numbers was observed.

The immune tolerance induction with methotrexate in Hurler syndrome (ITIMHS) study was necessarily limited by small patient numbers, as this was a prospectively conducted single-center study. However, as all participants would be expected to develop a high-titer antibody response without immune tolerance induction,[11] any reduction of antibody response below 1:4,000, previously shown to correlate with outcomes by Pal et al.,[12] could be considered clinically meaningful, even with small numbers.

Methotrexate monotherapy, therefore, does not appear to be an effective immune tolerance induction strategy in MPS I patients, though it appeared to be a promising strategy in both infantile Pompe disease patients[21,22] and in a mouse model of this disease.[19,20] A number of factors may contribute to this difference in efficacy. The antigens in each case (recombinant alfa-glucosidase and laronidase) were different and may have different degrees of immunogenicity, and the diseases may result in different responses in the host immune system.

In infantile Pompe disease, the absence of cross-reactive immunologic material (CRIM) is an important predictor of antibody responses. CRIM-negative status, as seen in patients with deleterious mutations and no detectable protein expression, is associated with the consistent development of high-titer antibodies and markedly poorer treatment outcomes.[27] However, a significant proportion of CRIM-positive infants also develop high-titer antibody responses with associated poor outcomes.[17] Immune tolerance induction regimens used in CRIM-negative infantile Pompe disease patients involve a combined approach with B cell depletion (rituximab) as well as methotrexate.[15,16] More recently, methotrexate monotherapy has been used to prevent antibody responses in small numbers of CRIM-positive patients, though only a proportion of these patients would have developed high sustained antibody titers without immunomodulation.[21,22]

In this study, all participants had clinical evidence of the severe form of the disease (MPS IH). In a longitudinal study of antibody

cellular uptake inhibition. No cellular uptake of laronidase inhibition was found in mice from the anti-CD4 monoclonal antibody-treated group (0.2%) (Figure 6A).

CD4 and Regulatory T Cell Populations in MPS I Mice

To determine whether treating mice with immune tolerance induction regimens modulates $CD4^+$ cell counts as well as regulatory T populations, splenocytes were harvested from all mice and analyzed using flow cytometry. No treatment groups showed significant differences in the percentage of $CD4^+$ cell compared to the non-injection group (ERT group, $p = 0.92$; methotrexate group, $p = 0.96$; monoclonal antibody group, $p = 0.20$; methotrexate + monoclonal antibodies group, $p = 0.12$; anti-CD4 monoclonal antibody group, $p = 0.96$; and anti-CD8 monoclonal antibody group, $p = 0.50$) (Figure 6B). There was also no significant difference in the percentage of regulatory T cells in treatment groups compared to the non-injection group (ERT group, $p = 0.72$; methotrexate group, $p = 0.99$; monoclonal antibody group, $p = 0.99$; methotrexate + monoclonal antibodies, $p = 0.48$ group; anti-CD4 monoclonal antibody group, $p = 0.99$; and anti-CD8 monoclonal antibody group, $p = 0.99$) (Figure S2).

DISCUSSION

To date, no immune tolerance induction regimen has been shown to successfully prevent the antibody response to ERT in MPS I patients. Attempts to prevent antibody responses to ERT in MPS I have so far been unsuccessful. An immune tolerance induction regimen based on cyclosporin A and azathioprine was successful in a canine model of MPS I, but subsequently it proved ineffective in patients.[8,26] In this study, a low-dose, short-course methotrexate monotherapy regimen did not prevent antibody production. All participants developed high-titer antibodies, and there was evidence of an IgM antibody response at week 4 in all participants, including one individual who continued the methotrexate regimen for 3 weeks, suggesting that this participant developed an antibody response even while receiving
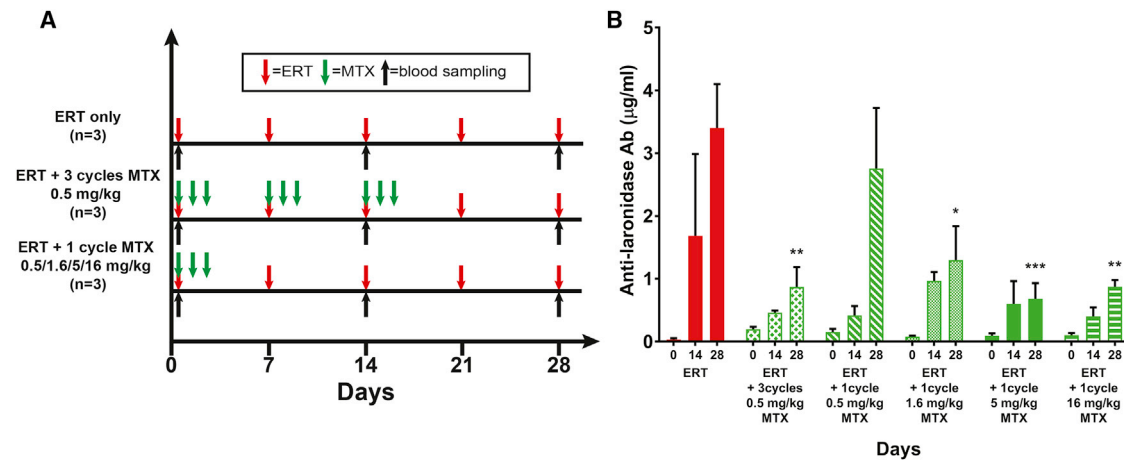
Figure 3. Methotrexate Dose Optimization Delivery Schedule and Antibody Levels in MPS I Mice

(A) All MPS I mice (n = 3 for each group) received 0.58 mg/kg laronidase weekly for 4 weeks. Some mice were given three cycles of 0.5 mg/kg methotrexate 0, 24, and 48 h after each laronidase treatment, and other mice received one cycle of methotrexate (doses ranged from 0.5 to 16 mg/kg) 0, 24, and 48 h after the first laronidase treatment. Blood samples were taken from all the mice on days 0, 13, and 28 for antibody analysis. (B) Serum anti-laronidase IgG antibodies were evaluated by ELISA. Data are shown as mean ± SEM, two-way ANOVA; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$ versus MPS I anti-laronidase IgG levels on day 28.

responses in eight MPS IH patients, all but one patient developed high-titer antibody responses during ERT, and the only patient that did not develop antibodies was under 3 months of age at the time of starting therapy.[11] Similarly, all patients with severe MPS I developed antibodies in the original clinical trial of ERT in patients under 5 years of age.[28] The majority of these patients had null mutations and would, therefore, be expected to be CRIM negative. The patients in our study would, therefore, reasonably be expected to develop antibodies without immunomodulation. However, it is interesting to note that only patient 1 in the study had two null mutations (Table 1). Though CRIM assays were not performed in this study, the mutations observed in patients 2 and 3 suggest that these patients may have produced some protein product, though without significant residual enzyme activity (Table 1). The missense mutation in patient 2 is reported to be associated with severe conformational changes and loss of stability of the protein product.[29] The in-frame deletion observed in patient 3 has been expressed in COS-7 cell lines, appears to result in a detectable protein product,[30] and is associated with very low enzyme activity; and, it is suggested that the mutation affects post-translational processing and trafficking to the lysosome.[31] The failure of methotrexate to prevent antibody responses in these patients, in comparison to its apparent success in CRIM-positive infantile Pompe disease patients, cannot, therefore, be solely ascribed to differences in CRIM status. However, it is important to note that, in the small study of low-dose methotrexate monotherapy in CRIM-positive infantile Pompe disease, 79% of methotrexate-treated patients only developed low antibody titers, but this was also the case for 68% of patients even without immunomodulation.[22] Patients with severe MPS I appear to be much more likely to generate strong antibody responses, even if their genotypes suggest they may be CRIM positive.

Immunogenicity to therapeutics in lysosomal disease remains an important clinical problem. In MPS I, HSCT is known to abrogate the antibody response, and, therefore, attenuated patients who do not receive HSCT but are treated with long-term ERT are the key subgroup of patients who may benefit from a successful immune tolerance induction strategy.[11,13] Prevention of an antibody response may also be relevant for MPS II, and the effects of antibodies need to be carefully investigated for new enzyme therapies, such as Sebelipase alfa for lysosomal acid lipase deficiency.[32–34] Given the different responses to similar immune tolerance induction regimens in infantile Pompe disease and MPS I patients, the ideal immune tolerance induction regimen for ERTs may differ between individual diseases and therapies. The relevance of the immune response is not limited to ERTs. With many gene therapies approaching or in clinical trial, immune responses to both vectors and protein products need to be considered. In addition, many patients will continue to be treated with standard ERTs before gene therapy approaches are widely available in clinical practice.

Effective strategies to prevent immune responses to therapies are, therefore, much needed to improve treatment outcomes in MPS I and potentially in other disorders. In this study, we went back to the MPS I mouse model to evaluate a number of potential immune tolerance induction strategies. One cycle of 5 mg/kg methotrexate was able to achieve a 5-fold reduction in antibody responses to laronidase by day 28 in the initial dose-response experiment and almost a 2-fold reduction (though not significant) at the equivalent time point in the later experiment. This suggests that methotrexate at a higher dose is capable of generating partial immune tolerance to laronidase; but, it is unable to abolish anti-laronidase antibody completely, and it is ineffective after adjuvant stimulation. This is similar to the results in
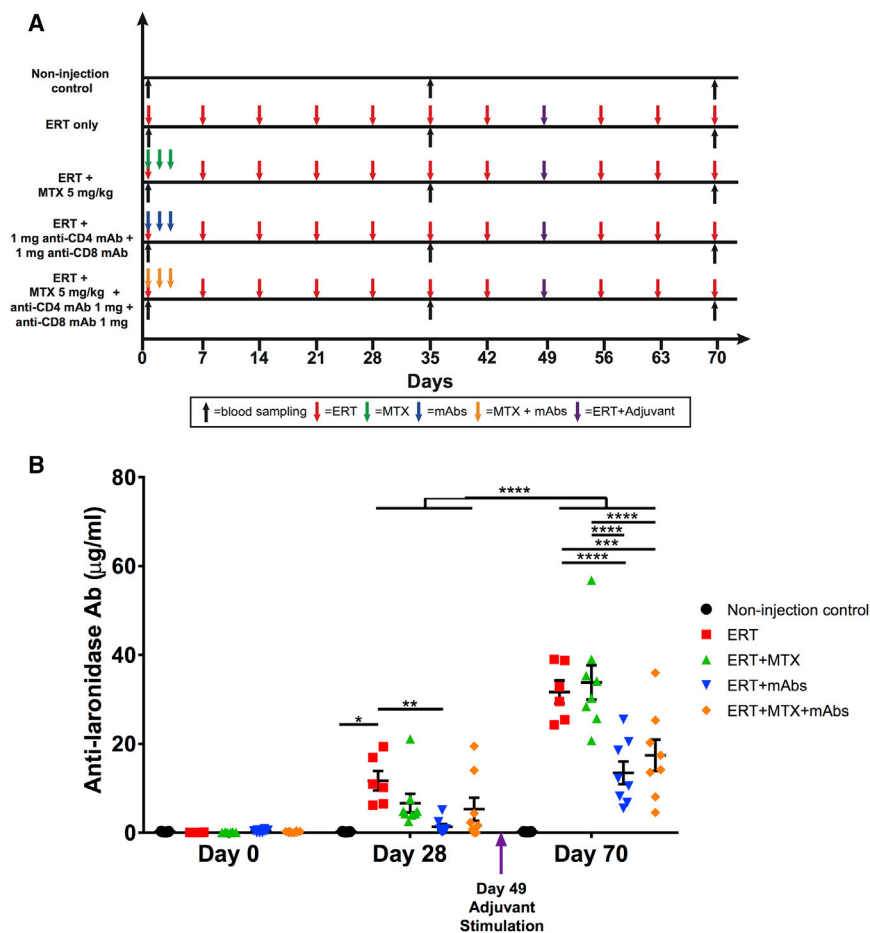
Figure 4. Anti-laronidase Antibodies in MPS I Mice Treated with Immune Tolerance Induction Regimens

(A) All MPS I mice from the treatment group (n = 8) received 0.58 mg/kg laronidase for 10 weeks. Laronidase delivered on day 49 was delivered in Sigma Adjuvant System (SAS). Immune tolerance induction (ITI) regimens were delivered to the mice (methotrexate, anti-CD4 and anti-CD8 monoclonal antibodies, and methotrexate with both monoclonal antibodies) 0, 24, and 48 h after the first laronidase treatment. Serum samples were taken on days 0, 28, and 70 for antibody analysis. Mice were sacrificed on day 70. (B) Anti-laronidase IgG antibody levels were measured in all serum samples using ELISA. Data are shown as mean ± SEM, two-way ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$, and ****$p < 0.00001$.

the Pompe mouse model in which methotrexate was able to diminish, but not eliminate, antibody responses to recombinant human glucosidase (rhGAA).[20] Methotrexate generates immune tolerance by interfering with cellular proliferation of rapidly dividing cells, such as T cells.[18] However, the very same property of methotrexate might have also hindered the development of regulatory T cells, which is crucial to immune tolerance generation. This is suggested by our results that MPS I mice treated with anti-CD4 and -CD8 monoclonal antibodies showed a 2.3-fold reduction in antibody responses but only achieved a 1.8-fold reduction when combined with methotrexate.

We demonstrated that the combination of non-depleting anti-CD4 and -CD8 monoclonal antibodies was able to reduce anti-laronidase antibody levels significantly, even after adjuvant stimulation, compared to the ERT controls. This suggests that the immune tolerance generated by the combination is relatively long lived, as the last dose of monoclonal antibodies was given 67 days prior to the last blood sampling time point. However, further improvement for this immune tolerance induction regimen was still much needed, as the anti-laronidase antibody levels produced in these mice were still significantly elevated compared to our non-injection controls. It was

also important to evaluate whether CD4 or CD8 has a more important role in inducing immune tolerance. For the second set of immune tolerance induction regimens, an extra cycle of either anti-CD4 monoclonal antibody or anti-CD8 monoclonal antibody was delivered to the mice before the adjuvant stimulation. Our results showed that two cycles of anti-CD4 monoclonal antibody was able to induce partial or complete immune tolerance to laronidase, even after adjuvant stimulation, while treatment with anti-CD8 monoclonal antibody was ineffective. The utility of a non-depleting anti-CD4 antibody in the reduction of antigen-specific antibodies was first reported by Qin et al.;[35] its ability to reduce immune tolerance to ERT was demonstrated in a Pompe mouse and when it was co-administered with factor VIII or IX in mice.[36,37]

Though we did not specifically investigate whether anti-CD4 mAb was able to abolish pre-existing anti-laronidase antibodies, we found that a second dose of anti-CD4 mAb was able to prevent antibody responses even after the huge stimulus of laronidase delivered alongside adjuvant. This result is encouraging as this may be a feasible immune tolerance induction regimen for attenuated MPS I patients who have developed immune responses to ERT but do not fit the criteria for a bone marrow transplant.

Non-depleting anti-CD4 monoclonal antibody may induce immune tolerance by the induction of regulatory T cells.[38,39] We therefore measured regulatory T cell populations in harvested spleens from MPS I mice. However, we were unable to detect any differences in regulatory T cell populations (Figure S2). This may have been due to limitations in our assay. Another possibility is that the immunomodulatory effects of the non-depleting anti-CD4 monoclonal antibody are mediated by a mechanism independent of regulatory T cells.[40,41]

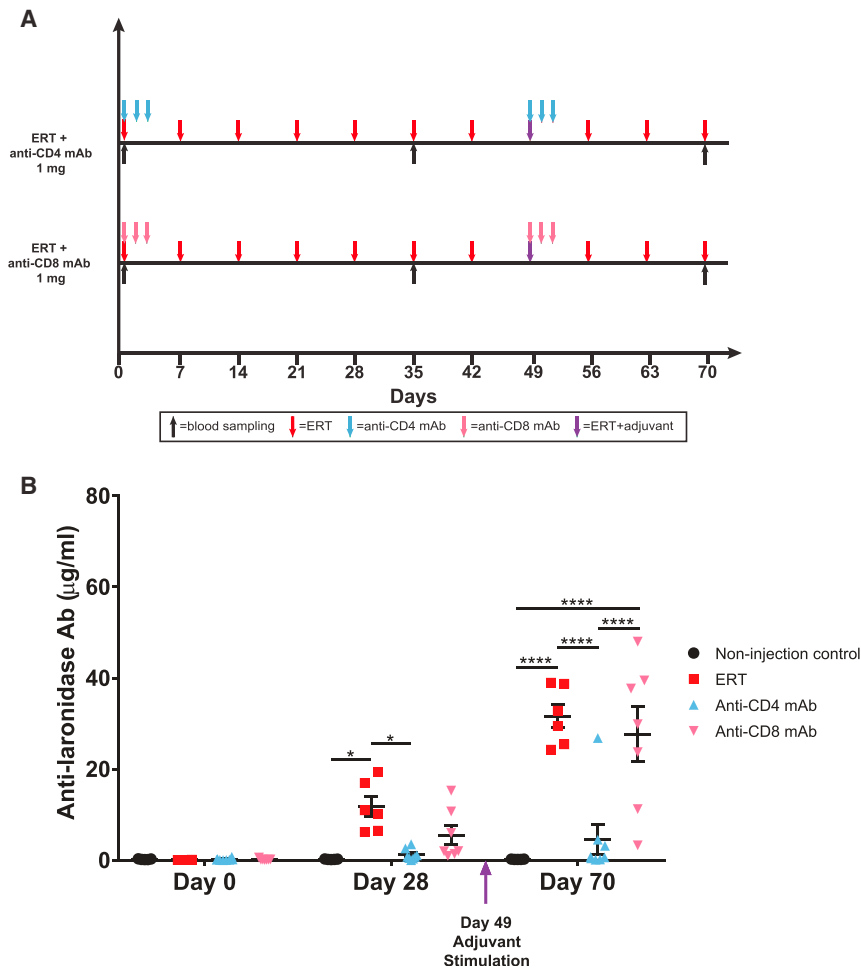**A**

B

compared to non-injection control groups. We did not specifically test the ability of immune tolerance induction treated mice to mount immune responses to non-specific antigens. However, previous studies investigating the use of non-depleting anti-CD4 monoclonal antibodies suggest that mice retain normal immune function. Mice treated with anti-CD4 monoclonal antibody that received skin allografts retained the ability to reject third-party grafts;[42] female mice receiving skin grafts from male mice alongside anti-CD4 monoclonal antibody cover were still able to mount proliferative T cell responses to the male antigen Dby;[38] mice receiving cardiac allografts alongside anti-CD4 monoclonal antibody were able to mount cytotoxic T lymphocyte responses to influenza virus;[43] and, in a mouse model of autoimmune arthritis, mice challenged with ovalbumin 30 days after treatment with anti-CD4 monoclonal antibody produced ovalbumin-specific immunoglobulin, but mice given ovalbumin at the same time as anti-CD4 monoclonal antibody treatment were unable to produce ovalbumin-specific immunoglobulin.[44] This suggests that tolerance is imposed only on antigens present at the time of tolerance induction.

Currently, a non-depleting anti-CD4 monoclonal antibody is not yet clinically available. The biologic agent tregalizumab has been in clinical development. This is a non-depleting IgG1 monoclonal antibody that binds to an epitope of CD4 and is reported to selectively induce the activation of regulatory T cells.[45] This was developed for psoriatic and rheumatoid arthritis (RA), and, although statistically significant efficacy for RA was not shown in a phase IIb study, the mechanism of action suggests that it may be a potentially useful agent for the prevention of antibody responses to therapeutic proteins. However, observations in this study suggest that a non-depleting anti-CD4 monoclonal antibody may have clinical applications in the management of antibody responses to therapeutic proteins. We suggest that evaluation of such a monoclonal antibody would be of

Inhibitory antibodies in MPS I patients are commonly found in those developing high antibody titers, and similar results were observed in our mouse study. MPS I mice from the ERT control group developed significant cellular uptake inhibition of the enzyme (70%), while the percentage of cellular uptake inhibition was significantly lower in all immune tolerance induction regimen-treated groups. MPS I mice receiving 2 cycles of anti-CD4 monoclonal antibody had no inhibitory antibodies, which was consistent with the IgG antibody level. The levels of inhibitory antibodies in the monoclonal antibodies- or combination of monoclonal antibodies and methotrexate-treated group were considered clinically insignificant (<30%), while inhibitory antibody levels of the methotrexate- and anti-CD8 monoclonal antibody-treated groups remained clinically significant.[12]

It is important that an immune tolerance induction regimen should not have a significant effect on normal immune function. Unlike experimental mice that were kept in individually ventilated cages, it is likely that patients will have frequent contact with sources of infection. We demonstrated that our immune tolerance induction regimens did not affect the number of CD4+ cells in all treated groups
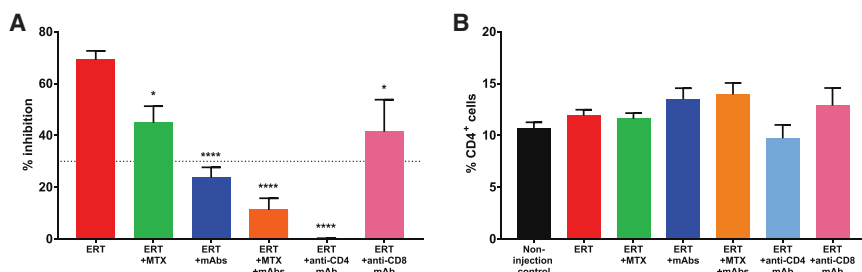
Figure 6. Cellular Uptake Inhibition and CD4$^+$ Cell Counts

(A) Cellular uptake inhibition of laronidase was measured in serum samples taken at endpoint (day 70) from all treated mice. Dotted line represents clinically relevant cellular uptake inhibition percentage (>30%). Data are shown as mean ± SEM, one-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.0001, and ****p < 0.00001. (B) CD4$^+$ cells in splenocytes were measured by flow cytometry.

benefit in MPS I, especially in attenuated patients, as well as in other LSDs where antibody responses to ERT is of clinical concern.

MATERIALS AND METHODS

Methotrexate Monotherapy in MPS IH Patients: Immune Tolerance Induction with Methotrexate in Hurler Syndrome Trial Design

The immune tolerance induction with methotrexate in Hurler syndrome study (EudraCT 2015-003031-35, REC 15/NI/0189) was a single-center, open-label trial investigating the safety and efficacy of methotrexate monotherapy in MPS IH patients. Informed consent was obtained from all subjects and the study was conducted in accordance with the principles set out in the WMA Declaration of Helsinki. Eligible participants were 3 months and 2.5 years old with a new diagnosis of MPS I and a classical severe (Hurler) phenotype who were eligible for HSCT and had not yet commenced ERT. All participants received a course of methotrexate in addition to standard clinical care (weekly ERT with laronidase prior to HSCT, as described by Ghosh et al.[5]). Participants received either 1 or 3 cycles of methotrexate. Each cycle comprised a series of 3 doses of oral methotrexate, given at 0.4 mg/kg/dose 60 min before laronidase infusion, 24 h after infusion, and 48 h after infusion (Figure 1).

Methotrexate dose selection was based on existing immune tolerance induction strategies used in infantile Pompe disease, in which a subcutaneous dose of 0.4 mg/kg has been used.[16] To minimize the invasiveness of the study intervention, oral methotrexate was selected over subcutaneous administration. The oral dose selected was based on pharmacokinetic modeling of this regimen performed as described by Ogungbenro and Aarons.[46] An oral dose of 0.4 mg/kg had similar pharmacokinetic properties to the subcutaneous dose (Figure S3), and the total weekly dose in infants is similar to the maximum licensed dose for children with inflammatory arthritis (25 mg/m^2).[47]

The primary outcome measure was anti-laronidase IgG antibody titers. Samples were collected at baseline (pre-ERT), week 4 of ERT, week 8 of ERT, immediately pre-HSCT, and 12 weeks post-HSCT. Additional outcome measures were anti-laronidase IgM titers, urinary GAGs, HS and DS in urine and serum, and T cell immunophenotyping. TEAEs were recorded throughout the study, though after the commencement of conditioning therapy for HSCT only TEAEs considered related to methotrexate were recorded (any serious TEAEs were recorded).

Anti-laronidase IgG and IgM Detection in Patient Serum by ELISA

Serum anti-laronidase IgG antibody titers were measured by the sandwich ELISA method described by Saif et al.[11] Briefly, 96-well enzyme immunoassay (EIA) plates were coated with 5 μg/mL laronidase (recombinant α-iduronidase; Genzyme, Cambridge, MA) diluted in coating buffer (1 M NaHCO$_3$ [pH 8.5]) overnight and blocked with blocking buffer containing 1% human serum albumin. Plates were washed three times with washing buffer PBS (Sigma-Aldrich, Gillingham, UK; 0.1% Tween), and 50 μL patient serum (in 2-fold serial dilutions) was added in duplicate. Plates were washed three times and incubated with either horseradish peroxidase-conjugated goat anti-human IgG antibody (Invitrogen, Camarillo, CA) at 1:5,000 dilution for 1 h or horseradish peroxidase-conjugated anti-human IgM antibody (Abcam, Cambridge, UK) at 1:20,000 dilution for 1 h. Plates were washed again and incubated with o-Phenylenediamine dihydrochloride substrate (Sigma-Aldrich, Gillingham, UK) for 10 min, and the reaction stopped with 2.5 M H$_2$SO$_4$. Plates were read immediately at 492 nm to determine the maximum absorbance and at 570 nm to correct for measurement errors. Anti-laronidase antibody titers were determined by defining a cutoff value in the dilution series by comparison of a patient sample to normal serum using the following formula:

$$\text{cutoff value} = (\text{mean absorbance of patient sample} - \text{SD})$$
$$- (\text{mean absorbance of normal serum} + 2 \times \text{SD})$$

Cellular Uptake Inhibition by Patient or Mouse Serum

Measurement of antibody-mediated inhibition of cellular uptake of enzyme was performed by the method described by Saif et al.[11] MPS I fibroblasts (MPS I-A171) from a patient with Hurler syndrome were maintained in culture medium (DMEM, 10% fetal bovine serum [FBS], and 1% glutamine; all from Sigma-Aldrich, Gillingham, UK) at 37°C and 5% CO$_2$. Six-well culture plates were seeded with fibroblasts at 1.5 × 10^6 cells/well and grown to 95% confluence. Laronidase was diluted in culture medium to a concentration of 100 ng/mL. Serum samples at a volume of 10 μL were added to 1,000 μL diluted enzyme and incubated for 2 h at room temperature. The culture medium was replaced with the enzyme-serum mix and incubated for 1 h at 37°C and 5% CO$_2$. Cells were washed with PBS and harvested, further washed, and resuspended in homogenization buffer (0.5 M NaCl and

0.02 M Tris [pH 7–7.5]). Following one freeze-thaw cycle, cells were sonicated at 5-μm amplitude and centrifuged at 2,045 × g for 10 min.

Enzyme activity was measured in cell lysates by a functional assay using 4-methylumbelliferyl-α-ʟ-iduronide substrate (Glycosynth, Warrington, UK), adapted from the method described by Stirling et al.[48] 20 μL cell lysate was added in duplicate to black 96-wel plates with 20 μL 4-methylumbelliferyl-α-ʟ-iduronide (4-MU) substrate (in substrate buffer, 0.4 M formate [pH 3.5] and 0.9% NaCl) and incubated for 1 h at 37°C in the dark. Enzyme activity was calculated by comparison to a standard curve constructed using serial dilutions of 4-MU fluorescent standard and stop solution (0.2 M NaCO₃ and 0.2 M NaHCO₃ in a 1:2 ratio [pH 9.5]). Enzyme activity measurements were corrected retrospectively for protein concentration in cell lysates performed by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA).

The percentage inhibition of cellular uptake inhibition (corrected for 100 μg lysate) was calculated as follows:

$$\% \text{ inhibition} = 100 - 100 \times \frac{\text{enzyme activity of lysate incubated with enzyme in the presence of patient serum}}{\text{enzyme activity of lysate incubated with enzyme only}}$$

Flow Cytometry Analysis of Patient PBMCs

T cell immunophenotyping was performed by flow cytometry on thawed peripheral blood mononuclear cells (PBMCs). PBMCs were isolated by Ficoll density gradient centrifugation of peripheral venous blood using Leucosep tubes (Greiner) and Histopaue-1077 (Sigma-Aldrich), as per the manufacturer's instructions. PBMCs were washed in PBS and resuspended in freezing medium (RPMI 1640 50%, 40% FBS, and 10% dimethyl sulfoxide), at a concentration of 10×10^6 cells/mL, before storage at −80°C. For immunophenotyping, PBMCs were thawed and washed in PBS twice before surface markers were labeled with the following: Zombie UV Live/Dead, CD3-AF700, CD14-APCCy7, CD19-APCCy7, HLA-DR-APCCy7, TCRγδ-PECF594, CD4-PB, CD127-BV785, CD25-PE (BioLegend, San Diego, CA), and CD56-PECy7 (eBioscience, San Diego, CA). Details of antibody clones and concentrations are given in Table S1.

After surface staining, PBMCs were fixed and permeabilized using BioLegend FoxP3 Fixation/Permeabilization Buffer set and stained with FoxP3-fluorescein isothiocyanate (FITC) (eBioscience, San Diego, CA). Cells were then washed with permeabilization buffer before immediate acquisition on a BD LSRFortessa instrument (BD Biosciences, San Jose, CA). At least 1×10^6 cells were acquired per sample. Voltage settings were standardized using single-stain positive and negative controls prepared using compensation beads (Comp-Beads, BD Biosciences). Samples were analyzed using FlowJo (version 10.3, FlowJo, Ashland, OR), and gating to determine percentage

positive expression was determined using the fluorescence-minus-one principle.

Urine GAG and DS:CS Ratio Analysis in Patient Samples

Urine GAGs were measured using the dimethylmethylene blue (DMB) assay based on the method first described by de Jong et al.[49] Urine samples or standards were mixed with DMB solution (Sigma-Aldrich), and absorbance was measured at 520 nm. As described by Whiteman,[50] two-dimensional electrophoresis of extracted glycosaminoglycans was performed on cellulose-acetate strips followed by staining with Alcian blue and washing. The DS:CS ratio was estimated based on the size and density of spots, as described by Church et al.[51]

HS and DS Analysis in Patient Serum and Urine

Concentrations of HS and DS in serum and urine were measured using the methods previously described by de Ru et al.[52] and Langereis et al.[10] HS and DS disaccharides were prepared by enzymatic digest and quantitated by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis.

Monoclonal Antibody Production

Some groups received non-depleting monoclonal antibodies that block CD4 (YTS177) and/or CD8 (YTS105), which were produced from hybridoma cell lines that were kindly provided by professor H. Waldmann, with protocol that was modified[25,35] from Honey et al.[53] Briefly, cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma-Aldrich, Gillingham, UK) with 5% FBS inside CELLine CL1000 flasks (Integra, Chur, Switzerland). The cell supernatant that contains the desired monoclonal antibody was harvested and purified using fractionated ammonium sulfate precipitation. Purified monoclonal antibody solutions were dialysed into PBS, concentrated using VivaSpin centrifugal concentration columns (Sartorius Stedim Biotech, Göttingen, Germany), and the final concentration of the monoclonal antibody was measured using Nanodrop at an absorbance of 180 nm. The purity and denaturation of the monoclonal antibody was tested by SDS and native PAGE. The endotoxin level of monoclonal antibody was measured using Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

MPS I Mouse Model

All procedures and mouse maintenance were carried out in accordance with UK Home Office regulations and guidelines in the Animals (Scientific Procedures) Act 1986 under project license P0C3AEEB0 held by B.W.B. B6.129-$Idua$^tm1Clk/J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA); the mutants

($Idua^{-/-}$) were produced by targeted disruption of murine *Idua* gene using homologous recombination in embryonic stem cells.[54] The mouse colony was maintained by inbreeding heterozygous mice on a C57BL/6 background. They were housed in a 12-h light-dark cycle in individually ventilated cages, with constant temperature control (21°C) and food and water supplied *ad libitum*.

ERT and Immune Tolerance Induction Regimens for MPS I Mice
Laronidase was delivered to MPS I mice intravenously weekly through the lateral tail vein, at a clinical dose of 0.58 mg/kg in 0.9% sterile saline.

For the methotrexate dose optimization experiment, MPS I mice (n = 3 each, 12–16 weeks old) received weekly laronidase for 3 weeks and either one or three cycles of methotrexate at 0.5–16 mg/kg (Calbiochem, San Diego, CA, USA) via intraperitoneal (i.p.) injection. A group of MPS I mice (n = 3) that received laronidase only was included as a positive control. Blood samples were taken from all mice on days 0, 13, and 28 for antibody analysis. Mice were euthanized on day 42.

For all other immune tolerance induction studies, all MPS I mice from treated groups (n = 8 per group, mixed sex) received weekly laronidase on weeks 1–6 and 8–10. On week 7, laronidase was diluted in 25 µL Sigma Adjuvant System (Sigma-Aldrich, Gillingham, UK) with 0.9% saline and delivered to the mice subcutaneously. MPS I mice from the first sets of immune tolerance induction regimen-treated groups received methotrexate (5 mg/kg) and/or 1 mg each of anti-CD4 and anti-CD8 monoclonal antibodies at 0, 24, and 48 h after the first dose of laronidase. MPS I mice from the second sets of immune tolerance induction regimen received either 1 mg anti-CD4 monoclonal antibody or 1 mg anti-CD8 monoclonal antibody at 0, 24, and 48 h after the first dose of laronidase and 0, 24, and 48 h after receiving the adjuvant stimulation. A group of untreated MPS I mice (n = 8) was used as a negative control. Blood samples were only taken from mice on days 0, 27, and 70 for antibody analysis; a blood sampling point on day 42 was added later for monitoring purposes as the experiment progressed. Mice were euthanized on day 70.

Anti-laronidase IgG in Mouse Serum by ELISA
Serum IgG anti-laronidase antibody levels were determined using a sandwich ELISA. Briefly, EIA plates were coated with 5 µg/mL laronidase diluted in coating buffer (0.1 M NaHCO$_3$ [pH 8.5]) overnight at 4°C. Plates were washed with wash buffer (PBS and 0.1% Tween) and blocked with blocking buffer (1% BSA, 0.02 M Tris/HCl, and 0.25 M NaCl) for 1 h at room temperature. The mouse anti-human IDUA antibody (antibodies-online.com, Aachen, Germany), standards, and the serum samples were diluted in dilution buffer (0.05% Tween and 0.01% BSA in PBS). Plates were washed; standards and serum samples were applied to the plate in duplicate for 1 h at room temperature. Plates were washed and incubated with biotinylated goat anti-mouse IgG antibody (Vector Laboratories, Peterborough, UK) at 5 µg/mL for 1 h at room temperature. The plates

were washed and then incubated with Vectastain ABC kit (Vector Laboratories, Peterborough, UK) for 30 min at room temperature, before incubating in 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific, Waltham, MA, USA) for exactly 3 min. 2.5 M H$_2$SO$_4$ was added to the plate to stop the reaction. Light absorbance was read at 450 nm to determine the maximum absorbance and at 570 nm to correct for measurement errors. The anti-laronidase IgG antibody concentrations of the serum samples were determined using the standard curve.

Flow Cytometry Analysis Splenocytes in MPS I Mice
T cell populations were accessed using flow cytometry using spleen samples harvested at endpoint. Single-cell suspensions of splenocytes were prepared, blocked (2% FBS and 5% mouse serum in PBS), and stained with anti-mouse CD4-FITC and anti-mouse CD25-antigen-presenting cell (APC)-Cy7. Surface-stained cells were fixed and permeabilized using BioLegend FoxP3 Fixation/Permeabilization Buffer Set and stained with anti-mouse FoxP3-PE (BioLegend, San Diego, CA, USA). Cells were washed with permeabilization buffer before acquisition on a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). Acquired data were analyzed using FlowJo version 10.3.

Statistics
Statistical analysis was performed using GraphPad Prism 7 software (La Jolla, CA, USA). In the immune tolerance induction with methotrexate in Hurler syndrome study, statistical analysis of biomarkers and regulatory T populations was performed using non-parametric repeated-measures analysis (Friedman), followed by Dunn's multiple comparisons test. Missing values were imputed using a last-observation-carried-forward method. For investigation of immune tolerance induction regimens in the MPS I mouse model, one-way or two-way ANOVAs were performed for multi-group analysis, followed by Tukey's multi-comparisons test. Significance was set at $p < 0.05$.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.02.007.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST

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