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Original Article

Targeting a Pre-existing Anti-transgene T Cell Response for Effective Gene Therapy of MPS-I in the Mouse Model of the Disease

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Mucopolysaccharidosis type I (MPS-I) is a severe genetic disease caused by a deficiency of the alpha-L-iduronidase (IDUA) enzyme. *Ex vivo* hematopoietic stem cell (HSC) gene therapy is a promising therapeutic approach for MPS-I, as demonstrated by preclinical studies performed in naive MPS-I mice. However, after enzyme replacement therapy (ERT), several MPS-I patients develop anti-IDUA immunity that may jeopardize *ex vivo* gene therapy efficacy. Here we treat MPS-I mice with an artificial immunization protocol to mimic the ERT effect in patients, and we demonstrate that IDUA-corrected HSC engraftment is impaired in pre-immunized animals by IDUA-specific $CD8^+$ T cells spared by pre-transplant irradiation. Conversely, humoral anti-IDUA immunity does not impact on IDUA-corrected HSC engraftment. The inclusion of lympho-depleting agents in pre-transplant conditioning of pre-immunized hosts allowes rescue of IDUA-corrected HSC engraftment, which is proportional to $CD8^+$ T cell eradication. Overall, these data demonstrate the relevance of pre-existing anti-transgene T cell immunity on *ex vivo* HSC gene therapy, and they suggest the application of tailored immune-depleting treatments, as well as a deeper immunological characterization of patients, to safeguard the therapeutic effects of *ex vivo* HSC gene therapy in immunocompetent hosts.

INTRODUCTION

In the last decades, viral vector-based gene therapy (GT) experienced a strong growth and turned into treatment of choice for rare inherited diseases, often allowing complete correction of symptoms.[1] Despite their therapeutic potential, the use of viral vectors is associated with immunological issues, which may impact on both the safety and efficacy of GT.

Among different strategies for therapeutic gene delivery to target cells, viral-derived particles are vectors of choice. However, being assembled with some structural proteins coming from parental viruses, they could potentially be recognized by a pre-alerted immune system, thus compromising the safety and efficacy of GT.[2–6] In this respect, the use of lentiviral vectors (LVs) is considered safe, since previous exposure of the patient to the parental virus is quite uncommon and easy to be predicted.[7] This, together with their ability to mediate stable integration of DNA into the host genome and to allow lifelong correction of the patient DNA,[8] made LVs powerful tools for GT applications. Thus far, LVs have been applied in different clinical trials based on *ex vivo* correction of autologous hematopoietic stem cells (HSCs), and they were demonstrated to be immunologically safe and therapeutically efficient in symptom correction.[9–11] Immunological concerns associated with GT are not restricted to anti-vector immunity. The transgene itself encodes for a therapeutic protein, which can be perceived as a foreign antigen by the immune system of null-mutation subjects. Anti-transgene immunity is known to be induced *de novo* after *in vivo* GT with LVs.[12] This results from the simultaneous exposure of the host to a novel antigen and to virally driven mediators of innate immunity. Conversely, *ex vivo* transduction of therapeutic cells avoids direct exposure of the patient to viral particles, limiting immune activation.

Ex vivo HSC GT recently was demonstrated to be a powerful therapeutic strategy for the lysosomal storage disorder (LSD) metachromatic leukodystrophy (MLD), displaying a good safety profile and

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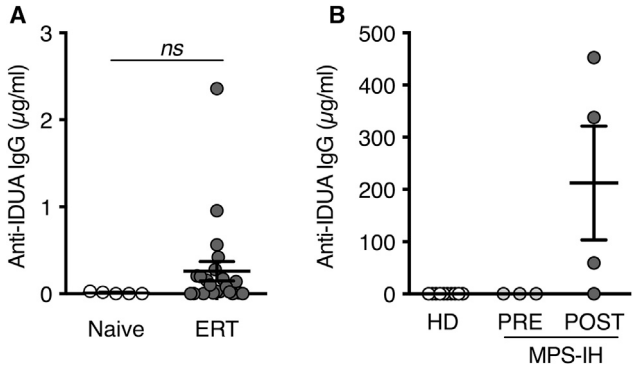

Figure 1. Enzyme Replacement Therapy with rhIDUA (Aldurazyme) Is Poorly Immunogenic in MPS-I Mice

(A) MPS-I mice were either left untreated (naive, n = 5) or weekly i.v. injected with rhIDUA (ERT, n = 22). At 4 weeks after treatment, the concentration of anti-IDUA IgGs in serum was measured by ELISA. Each dot represents one mouse; error bars indicate mean ± SEM. Mann-Whitney test. (B) The concentrations of anti-IDUA IgGs in the sera of healthy donors (HD, n = 10) and MPS-IH patients, before (PRE, n = 3) and after (POST, n = 4) ERT, were measured by ELISA. Each dot represents one subject; error bars indicate mean ± SEM.

arresting disease progression when applied in pre-symptomatic patients.[10,11] This provided a strong rationale for translating the *ex vivo* HSC GT platform to other LSDs, including Mucopolysaccharidosis type I (MPS-I), which results from the lack or impaired activity of the alpha-L-iduronidase (IDUA) enzyme. In the absence of IDUA catabolic activity, enzyme substrates progressively accumulate in soft and connective tissues, resulting in severe impairment of organ function and premature death.[13] The severe form of the disease (Hurler syndrome) is currently treated with allogeneic HSC transplantation (HSCT), which, despite having improved the morbidity and quality of life of patients, leaves them with a significant disease burden, especially in the CNS and bones.[14]

This provided the rationale for testing alternative transplantation strategies, such as HSC GT approaches. It was proven that naive MPS-I mice transplanted with autologous *ex vivo* IDUA-corrected HSCs benefit from a therapeutic advantage significantly higher than allogeneic HSCT.[15] Accordingly, this platform is currently under clinical evaluation in a phase I/II clinical trial opened at San Raffaele Scientific Institute for MPS-I Hurler (MPS-IH) patients (ClinicalTrials.gov: NCT03488394). However, enzyme replacement therapy (ERT) is currently recommended after MPS-I diagnosis to slow down disease burden, improve clinical outcome, and reduce the morbidity of allogeneic HSCT.[16,17] Similar to other pathological settings resulting from null mutations, the immune system of MPS-IH patients recognizes IDUA as a foreign antigen, resulting in anti-IDUA immunoglobulin G (IgG) production in 91% of treated subjects.[18,19] The impact of pre-existing anti-enzyme immunity on *ex vivo* HSC GT has been poorly studied so far; thus, we investigated if therapeutic IDUA-transduced HSCs expressing supra-physiological levels of the enzyme may be selectively targeted by ERT-induced anti-IDUA immunity.

In this study, we optimize an artificial immunization protocol to induce in MPS-I mice a strong and homogeneous anti-IDUA immune response, and we show that IDUA-corrected HSCs do not engraft in pre-immunized MPS-I mice. While pre-existing anti-IDUA IgGs do not impact on *ex vivo* HSC GT, IDUA-specific $CD8^+$ T cells mediate the clearance of IDUA-corrected HSCs. Effective depletion of the T cell compartment rescues the engraftment of IDUA-corrected cells in pre-immunized MPS-I mice. Interestingly, a simultaneous stimulation of the innate immune response, such as concomitant tissue damage or administration of a Toll-like receptor (TLR)3 agonist, dramatically increases the anti-IDUA immune response in ERT-treated MPS-I mice.

This study highlights the safety and efficacy issues deriving from pre-existing anti-transgene immunity in *ex vivo* HSC GT settings. Accordingly, ERT-induced cellular immunity in immunocompetent subjects who are candidates for GT should be deeply characterized and carefully monitored before and after the transplantation of gene-corrected HSCs.

RESULTS

Induction of Anti-IDUA Immune Response in MPS-I Mice

To mimic in the preclinical model of the disease the effects of ERT in MPS-IH patients, recombinant human IDUA (rhIDUA) was intravenously (i.v.) injected once a week (0.58 μg/g) in *idua*$^{-/-}$ (MPS-I) mice (ERT, n = 22). After five injections, IDUA-specific IgGs were detectable in 12 of 22 treated mice (ranging from 0.079 to 2.358 μg/mL) (Figure 1A), while T cells isolated from the spleen of ERT-treated mice did not proliferate in response to rhIDUA *in vitro* (data not shown). ERT-treated and control naive MPS-I mice were lethally irradiated and transplanted with bone marrow (BM)-derived autologous HSCs transduced *in vitro* with LV encoding for human IDUA (LV.IDUA), as previously described.[15] IDUA-transduced HSCs engrafted with the same efficiency in ERT-treated and naive MPS-I mice (data not shown), indicating that the pre-existing anti-IDUA response induced by ERT in MPS-I mice does not impair efficacy of *ex vivo* GT. However, in the sera of MPS-IH patients receiving ERT, we measured a 1,000-fold higher concentration of anti-IDUA IgGs (Figure 1B) compared to that observed in ERT-treated MPS-I mice (Figure 1A): on average 213 μg/mL in patients versus 0.26 μg/mL in mice. These data suggest that the ERT model we used may not be suitable to dissect the impact of pre-existing anti-IDUA immunity on *ex vivo* HSC GT for MPS-I.

To promote a stronger pre-existing anti-IDUA immunity in MPS-I mice, we developed an experimentally induced anti-IDUA immunity model. Specifically, MPS-I mice were immunized (IDUA-IMM) with rhIDUA in incomplete Freund's adjuvant (IFA) and developed anti-IDUA IgGs (23.3 ± 18.95 μg/mL, mean ± SEM; n = 21; Figure 2A). *Ex vivo* analyses revealed the presence of B and plasma cells actively secreting anti-IDUA IgGs in the spleen and BM of IDUA-IMM mice (5.8 ± 6.4 and 2.5 ± 4 spot-forming units [SFU]/3.5 × 10^5 cells, mean ± SEM; n = 6, respectively; Figure 2B). Moreover, significantly

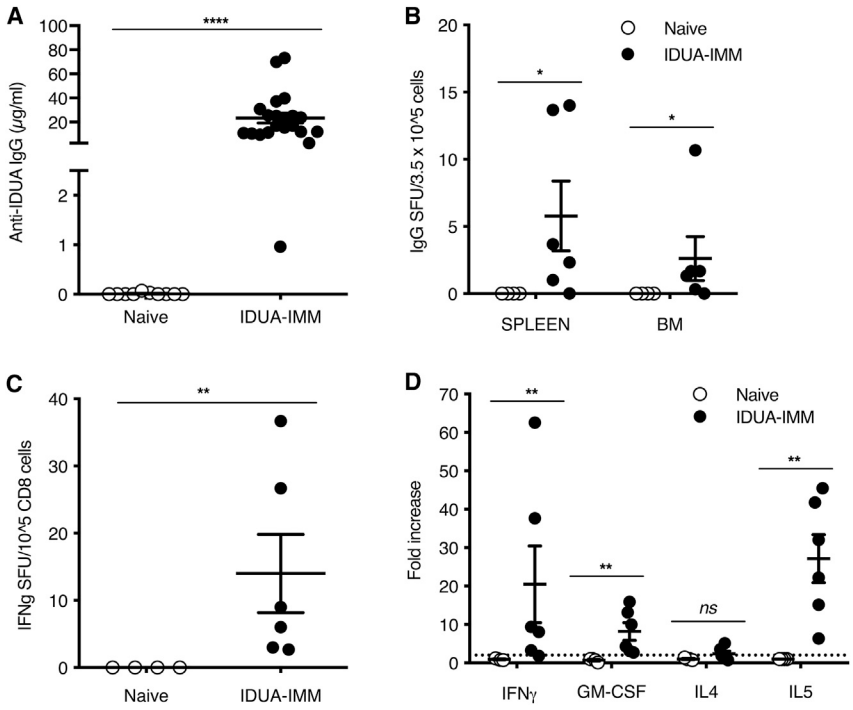

Figure 2. IDUA-IMM Mice Develop an Anti-IDUA Immune Response

(A) MPS-I mice were either left untreated (naive, n = 10) or immunized with rhIDUA in Incomplete Freund's Adjuvant (IDUA-IMM, n = 21). At 35 days after immunization, mice were sacrificed and the concentration of anti-IDUA IgGs in serum was measured by ELISA. Each dot represents one mouse; error bars indicate mean ± SEM. ****$p \leq 0.00005$, Mann-Whitney test. (B) Numbers of B and plasma cells actively secreting anti-IDUA IgGs were quantified by ELISPOT assay as spot-forming units (SFU)/3.5 × 10^5 total cells isolated from the spleen and BM of mice. Values for mean ± SEM of naive (n = 4) and IDUA-IMM (n = 6) MPS-I mice are shown. *$p \leq 0.05$, Mann-Whitney test. (C) Number of IDUA-specific $CD8^+$ T cells was measured by ELISPOT assay as SFU/10^5 $CD8^+$ T cells isolated from the spleen of treated mice. Each dot represents one mouse; error bars indicate mean ± SEM of naive (n = 4) and IDUA-IMM (n = 6) MPS-I mice. **$p \leq 0.005$, Mann-Whitney test. (D) Total splenocytes were stimulated with rhIDUA (Aldurazyme, 10 μg/mL), and cytokine concentration was measured in culture supernatants at day 4 by Bioplex assay. Results are expressed as fold increase of cytokine secretion in stimulated compared to not-stimulated conditions. Values for mean ± SEM of naive (n = 4) and IDUA-IMM (n = 6) MPS-I mice are shown. **$p \leq 0.005$, Mann-Whitney test.

high numbers of anti-IDUA $CD8^+$ T cells secreting interferon (IFN)γ were detected in the spleen of IDUA-IMM mice (14 ± 5.81 SFU/10^5 cells, mean ± SEM) (Figure 2C), and splenocytes from IDUA-IMM mice secreted IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, and IL-5 upon *in vitro* stimulation with rhIDUA protein (Figure 2D).

In conclusion, immunization of MPS-I mice with rhIDUA in IFA homogeneously induces a strong anti-IDUA humoral immunity, which is associated with IDUA-specific T cell responses.

Pre-existing Anti-IDUA Immunity Prevents Engraftment of IDUA-Transduced HSCs in Immunized MPS-I Mice in an Antigen-Specific Manner

To evaluate the impact of pre-existing anti-IDUA immunity on *ex vivo* HSC GT for MPS-I, we transplanted naive and IDUA-IMM MPS-I mice with LV.IDUA-transduced HSCs (IDUA-GT) or LV.OVA-transduced HSCs (OVA-GT), and we monitored their engraftment (Figure 3A). IDUA-IMM IDUA-GT mice died in 3 weeks with the same kinetic of mice irradiated and not transplanted (Irrad Ctrl, n = 8) as a consequence of engraftment failure, as confirmed by vector copy number (VCN) quantification in BM (Figures 3B and 3C). Conversely, LV.IDUA-transduced HSCs efficiently engrafted in naive MPS-I mice, as previously shown.[15] Interestingly, LV.OVA-transduced cells engrafted both in IDUA-IMM and naive MPS-I mice (Figures 3B and 3C), indicating that the clearance of LV.IDUA-transduced HSCs in IDUA-IMM mice occurred in an antigen-specific fashion. IDUA-specific IgGs were detected at a high concentration in the sera of IDUA-IMM mice before and after IDUA-GT and OVA-GT (Figure 3D). Moreover, 12 weeks post-transplantation, B cells actively secreting anti-IDUA IgGs (Figure 3E) and IDUA-specific $CD8^+$ T cells (Figure 3F) were still detectable in the spleen of IDUA-IMM OVA-GT mice.

To exclude that rejection of transduced cells occurred as a consequence of IFA-mediated activation of innate immunity, MPS-I mice immunized with IFA emulsified with PBS (IFA-PBS) were lethally irradiated and transplanted with LV.IDUA-transduced HSCs. Results showed that IDUA-transduced HSCs engrafted in IFA-PBS mice, but not in IDUA-IMM mice (Figure S1A). Moreover, anti-IDUA IgGs were absent in IFA-PBS, but not in IDUA-IMM, MPS-I mice 2 weeks after transplantation (Figure S1B), and no IDUA-specific $CD8^+$ T cells were detected in the spleen of IFA-PBS mice 12 weeks after transplantation (data not shown).

These results demonstrate a detrimental effect of pre-existing anti-IDUA immunity on the engraftment of IDUA-transduced HSCs in MPS-I mice, and they show the persistence of anti-IDUA humoral and cellular responses in immunized MPS-I mice after irradiation and HSC transplantation.

Pre-existing Anti-IDUA IgGs Do Not Prevent Engraftment of IDUA-Transduced HSCs in Pre-immunized MPS-I Mice

To elucidate the role of anti-IDUA IgGs in the inhibition of IDUA-transduced HSC engraftment in IDUA-IMM mice, total IgGs isolated from the sera of MPS-I mice immunized with IDUA or OVA (IDUA-IMM or OVA-IMM) were transferred into naive MPS-I mice. IgG-treated and control naive MPS-I mice were then irradiated and

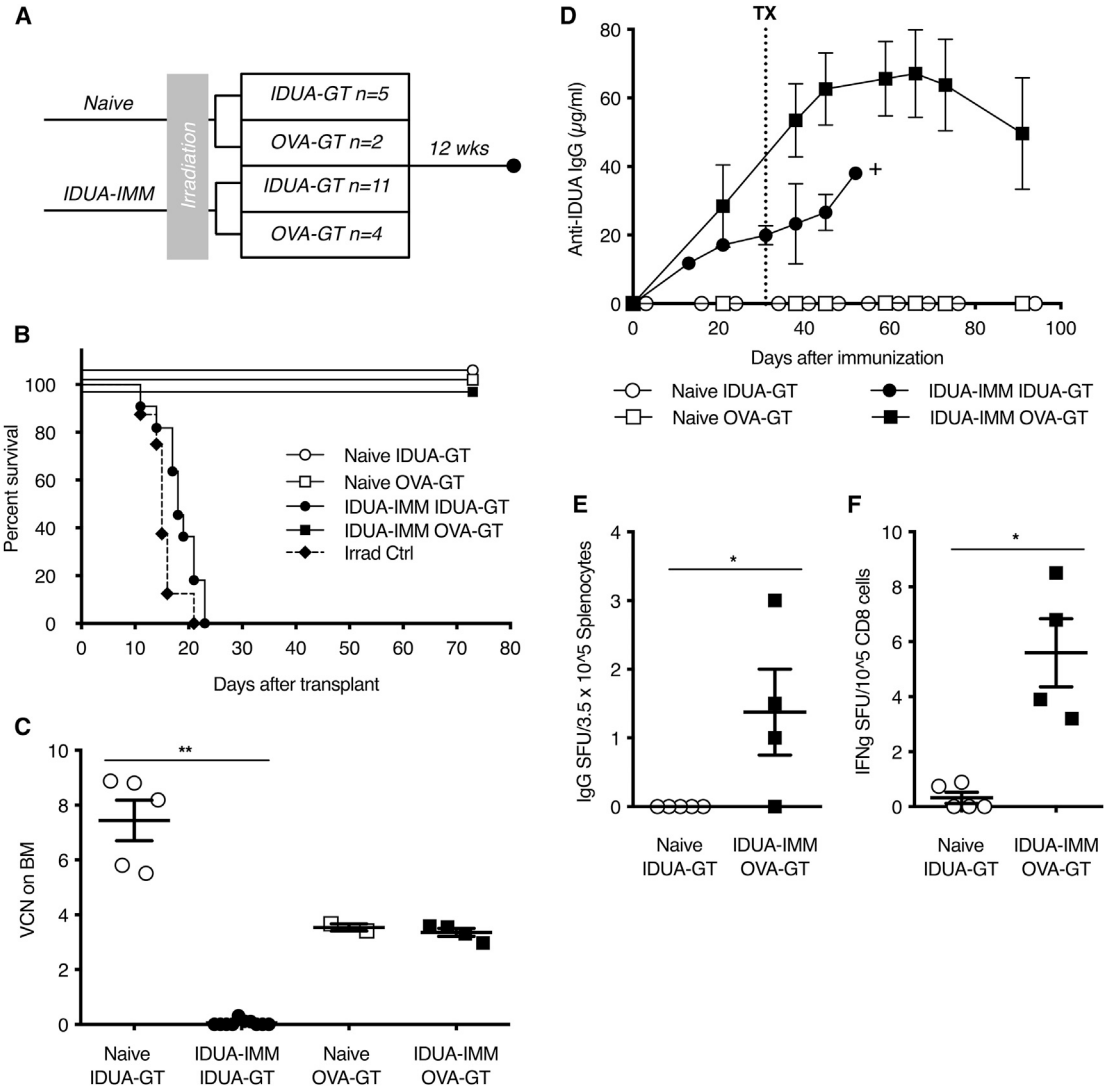

Figure 3. Pre-existing Anti-IDUA Immunity Prevents Engraftment of IDUA-Transduced HSCs in an Antigen-Specific Manner

(A) Naive and IDUA-IMM MPS-I mice were lethally irradiated and i.v. injected with LV.IDUA (IDUA-GT)- or LV-OVA (OVA-GT)-transduced autologous BM-derived HSCs (10^6 cells/mouse). (B) Percentages of survival for naive MPS-I mice transplanted with LV.IDUA-transduced HSCs (naive IDUA-GT, n = 5), naive MPS-I mice transplanted with LV.OVA-transduced HSCs (naive OVA-GT, n = 2), IDUA-IMM MPS-I mice transplanted with LV.IDUA-transduced HSCs (IDUA-IMM IDUA-GT, n = 11), IDUA-IMM MPS-I mice transplanted with LV.OVA-transduced HSCs (IDUA-IMM OVA-GT, n = 4), and irradiated and not transplanted MPS-I mice (Irrad Ctrl, n = 8). (C) At 12 weeks after transplantation, vector copy number was quantified by digital droplet PCR (ddPCR) in BM cells (VCN BM) of treated mice. Each dot represents one mouse; error bars indicate mean ± SEM. **$p \leq 0.005$, Mann-Whitney test. (D) The concentration of anti-IDUA IgGs in serum was measured by ELISA before and after transplantation. Results are shown as mean ± SEM. (E) At 12 weeks after transplantation, the number of anti-IDUA IgG-secreting cells was quantified by ELISPOT assay as SFU/3.5 × 10^5 total splenocytes. Values for mean ± SEM of naive (n = 5) and IDUA-IMM OVA-GT (n = 4) MPS-I mice are shown. *$p \leq 0.05$, Mann-Whitney test. (F) At 12 weeks after transplantation, the number of IDUA-specific $CD8^+$ cells was measured by ELISPOT assay as SFU/10^5 $CD8^+$ T cells isolated from the spleen of treated mice. Each dot represents one mouse; error bars indicate mean ± SEM of naive (n = 5) and IDUA-IMM (n = 4) MPS-I mice. *$p \leq 0.05$, Mann-Whitney test.

transplanted with IDUA-transduced HSCs. All transplanted mice survived (Figure 4A). LV-transduced HSCs engrafted in IgG-treated MPS-I mice, and the VCN quantified in BM and peripheral blood cells was comparable among the groups, regardless of the antigen specificity of the injected IgGs (Figure 4B). Moreover, anti-IDUA and anti-OVA IgGs were detected in the sera of transplanted mice before and after GT (Figure 4C).

These findings demonstrate that the presence of pre-existing IDUA-specific IgGs neither impairs the engraftment of IDUA-transduced

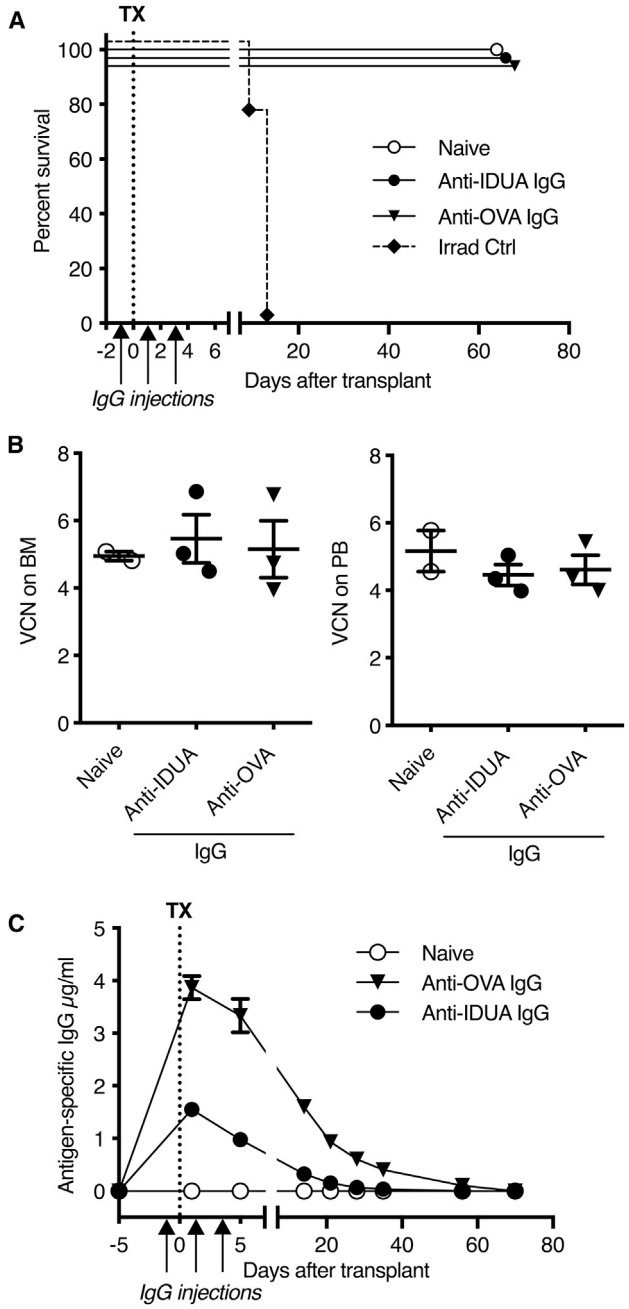

Figure 4. Anti-IDUA IgGs Do Not Prevent Engraftment of IDUA-Transduced HSCs in MPS-I Mice

Naive MPS-I mice were left untreated (naive, n = 2) or were i.v. injected with anti-IDUA IgGs (n = 3) or anti-OVA IgGs (n = 3) on days −1, +1, and +3 from transplantation of LV.IDUA-transduced autologous BM-derived HSCs (10^6 cells/mouse). (A) Percentages of survival. (B) At 12 weeks after transplantation, vector copy number was quantified by ddPCR in the peripheral blood (VCN PB) and BM (VCN BM) cells of treated mice. Each dot represents one mouse; error bars indicate mean ± SEM. (C) The concentrations of anti-IDUA and anti-OVA IgGs were measured by ELISA in the sera of treated MPS-I mice before and after transplantation. Results are shown as mean ± SEM.

HSCs in BM nor mediates the selection of untransduced HSCs in the periphery.

Pre-existing IDUA-Specific CD8$^+$ T Cells Impair Engraftment of IDUA-Transduced HSCs in Pre-immunized MPS-I Mice

To investigate the effects of anti-IDUA T cell responses on *ex vivo* HSC GT, LV.IDUA-transduced (CD45.1) and untransduced (CD45.2) HSCs were transplanted at a 1:1 ratio in IDUA-IMM or OVA-IMM mice (Figure S2A). The engraftment of untransduced HSCs allowed the survival of treated mice (Figure S2B) and the analysis of anti-IDUA T cell responses at endpoint in all groups. In IDUA-IMM, but not in OVA-IMM, mice, IDUA-transduced cells were selectively eliminated (Figure S2C). At 12 weeks post-transplantation in the spleen of IDUA-IMM mice, we identified a high frequency of anti-IDUA CD8$^+$ T cells (127 ± 26 SFU/10^5 cells, mean ± SEM; n = 7; Figure S2D), which was 10-fold higher than that detected in IDUA-IMM mice not transplanted or transplanted with OVA-transduced HSCs (Figures 2C and 3F, respectively). These data, together with the observation that, in IDUA-IMM mice, a significant percentage of CD8$^+$ T cells persists in the peripheral blood after lethal irradiation (Figure S3), suggest that residual IDUA-specific CD8$^+$ T cells may be re-activated and expanded after exposure to IDUA-overexpressing HSCs, thus potentially affecting the engraftment of *ex vivo* HSC GT in IDUA-IMM mice.

To confirm that IDUA-specific CD8$^+$ T cells affect the engraftment of IDUA-transduced HSCs, we treated IDUA-IMM IDUA-GT mice with anti-CD8-depleting monoclonal antibody (mAb). Untreated IDUA-IMM and naive MPS-I mice transplanted with LV.IDUA-transduced HSCs served as positive and negative controls, respectively (Figure 5A). At the time of transplantation, CD8$^+$ T cells were completely depleted from the peripheral blood of anti-CD8-treated IDUA-IMM mice (Figure 5B), and, to ensure continuous CD8$^+$ T cell ablation, anti-CD8 mAb was weekly injected in mice after transplantation. Depletion of CD8$^+$ T cells allowed the survival of IDUA-IMM IDUA-GT mice (Figure 5C) and the engraftment of IDUA-transduced HSCs (Figure 5D). Anti-IDUA IgGs, regardless of anti-CD8 treatment, were detected over time in the sera of IDUA-IMM IDUA-GT mice (Figure 5E), confirming that the presence of anti-IDUA IgGs does not impair the engraftment of IDUA-transduced HSCs.

These data show that pre-existing IDUA-specific CD8$^+$ T cells prevent the engraftment of IDUA-transduced HSCs in pre-immunized MPS-I mice.

T Cell-Depleting Treatments Partially Rescue Engraftment of IDUA-Transduced HSCs in Pre-immunized MPS-I Mice

To eradicate pre-existing anti-IDUA CD8$^+$ T cells, we tested two different T cell depleting agents: Fludarabine and anti-CD3 mAb. We first treated MPS-I mice with 600, 800, or 1,000 µg/g Fludarabine intraperitoneally to quantify the dose-dependent lympho-depletive effect. The highest tolerated dose, providing 50% depletion of circulating CD3 cells, was chosen for further investigations (Figure S4).

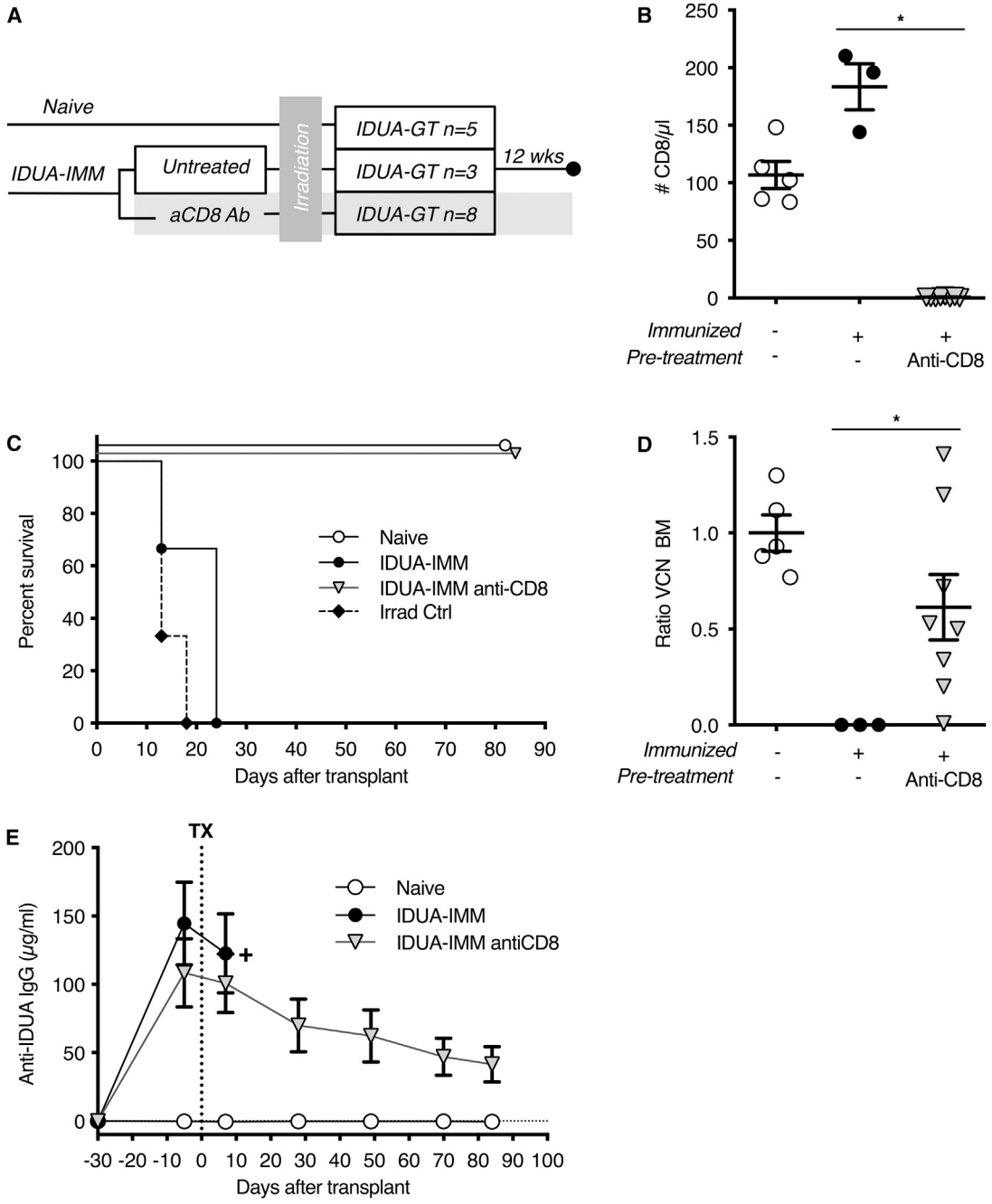

Figure 5. CD8$^+$ T Cell Depletion Rescues Engraftment of IDUA-Transduced HSCs in Immunized MPS-I Mice

(A) Naive and IDUA-IMM MPS-I mice left untreated or treated with anti-CD8 depleting mAb were lethally irradiated and transplanted with LV.IDUA-transduced (10^6 cells/mouse) autologous BM-derived HSCs. (B) At the time of transplantation, the absolute number of CD8$^+$ T cells was quantified in the peripheral blood of treated mice by fluorescence-activated cell sorting (FACS) staining. Each dot represents one mouse; error bars indicate mean ± SEM. *$p \leq 0.05$, Mann-Whitney test. (C) Percentages of survival for naive mice transplanted with LV.IDUA-transduced cells (naive, n = 5), IDUA-IMM MPS-I mice transplanted with LV.IDUA-transduced cells untreated (IDUA-IMM n = 3) or treated with anti-CD8 mAb (IDUA-IMM anti-CD8, n = 8), and irradiated and not-transplanted MPS-I mice (Irrad Ctrl, n = 3). (D) At 12 weeks after transplantation, vector copy number was quantified by ddPCR in BM cells (VCN BM) of treated mice. Each dot represents absolute VCN of a single mouse normalized on the mean of VCN of the naive group; error bars indicate mean ± SEM. *$p \leq 0.05$, Mann-Whitney test. (E) The concentration of anti-IDUA IgGs was measured by ELISA in the sera of treated MPS-I mice before and after transplantation. Results are shown as mean ± SEM.

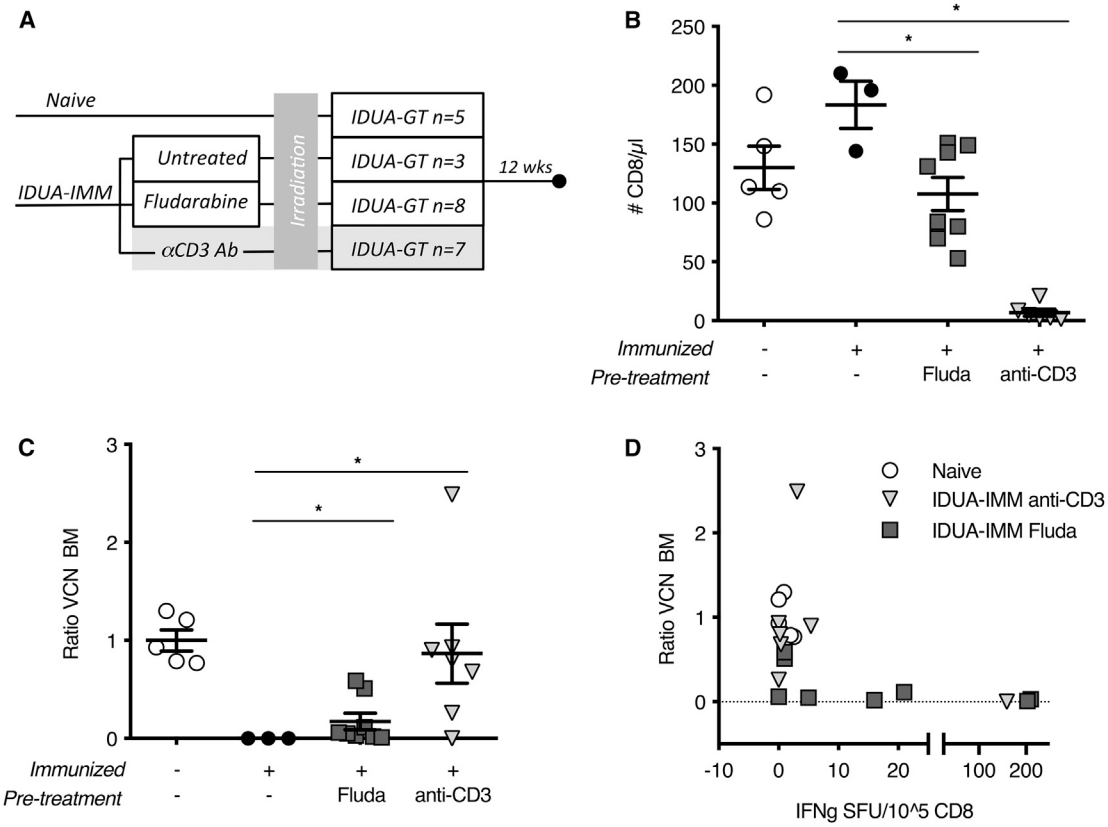

Figure 6. Pre-treatment with T Cell Depleting Agents Partially Rescues Engraftment of IDUA-Transduced HSCs in Immunized MPS-I Mice

(A) Naive and IDUA-IMM MPS-I mice left untreated or treated with Fludarabine or anti-CD3 depleting mAb were lethally irradiated and transplanted with LV-IDUA-transduced BM-derived HSCs isolated from autologous mice (IDUA-GT, 10^6 cells/mouse). (B) At the time of transplantation, the absolute number of $CD8^+$ T cells was quantified in peripheral blood of treated mice by FACS staining. Each dot represents one mouse; error bars indicate mean ± SEM. *$p \leq 0.05$, Mann-Whitney test. (C) At 12 weeks after transplantation, vector copy number was quantified by ddPCR in BM cells (VCN BM) of treated and control MPS-I mice. Each dot represents absolute VCN of a single mouse normalized on the mean of VCN of the naive group; error bars indicate mean ± SEM. *$p \leq 0.05$, Mann-Whitney test. (D) At 12 weeks after transplantation, the number of IDUA-specific $CD8^+$ T cells was quantified by ELISPOT assay as SFU/10^5 $CD8^+$ T cells isolated from the spleen of treated mice. The number of IDUA-specific $CD8^+$ T cells was correlated to the VCN of a single mouse normalized on the mean of VCN of the naive untreated group VCN data. Each dot represents one mouse.

IDUA-IMM mice were left untreated or treated with Fludarabine or anti-CD3 mAb and subsequently lethally irradiated and transplanted with IDUA-transduced HSCs. Naive MPS-I mice were irradiated and transplanted in parallel as positive controls of engraftment (Figure 6A). At the time of transplantation, a complete depletion of $CD8^+$ T cells from the peripheral blood of IDUA-IMM mice was induced by anti-CD3 mAb, while treatment with Fludarabine partially depleted $CD8^+$ T cells (Figure 6B). Accordingly, IDUA-transduced HSCs engrafted more efficiently in IDUA-IMM mice treated with anti-CD3 (6 of 7) than in Fludarabine-treated IDUA-IMM mice (2 of 8) (Figure 6C). At 12 weeks after transplantation, analysis of IDUA-specific $CD8^+$ T cells in the spleen of treated mice demonstrated their persistence and expansion in those mice in which IDUA-transduced HSCs were completely eliminated (Figure 6D).

These observations confirm the critical role played by pre-existing anti-IDUA $CD8^+$ T cells in preventing the engraftment of IDUA-transduced HSCs, and they demonstrate that, prior to *ex vivo* HSCT GT, a profound $CD8^+$ T cell depletion is required to favor efficient engraftment of gene-corrected cells in IDUA-IMM mice.

Concomitant Activation of Innate Response Promotes IDUA-Specific T Cells in MPS-I Mice Receiving ERT

ERT treatment is poorly immunogenic in MPS-I mice (Figure 1A), however, it is known that simultaneous stimulation of the innate immune system by danger signals is needed to promote adaptive immunity against exogenous proteins. We therefore administered ERT to MPS-I mice either intramuscularly (i.m.) to generate a tissue damage or i.v. in combination with poly(I:C) to promote innate immunity activation through TLR3 stimulation (ERT-poly(I:C)) and simulate an undergoing viral infection. IDUA-specific IgGs and $CD8^+$ T cells were induced in both i.m. and ERT-poly(I:C) mice, but not, as expected, in the ERT control group (Figures S5A and S5B). Since MPS-I patients under ERT may undergo viral infections,

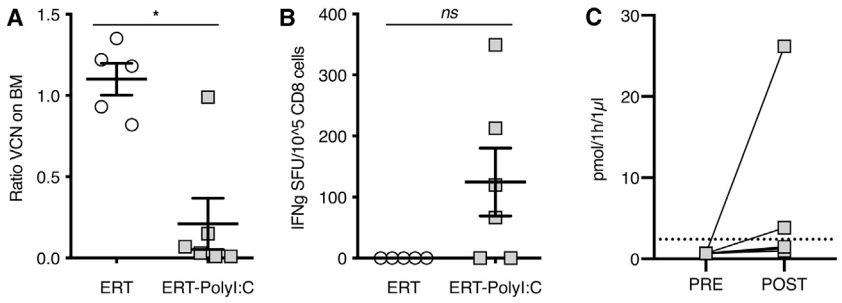

Figure 7. Viral Infection Simulation during ERT Induces Anti-IDUA CD8⁺ T Cells Able to Eliminate LV.IDUA-Transduced HSCs

MPS-I mice received 5 weekly i.v. injections of rhIDUA alone (ERT, n = 5) or in combination with poly(I:C) on weeks 2 and 3 (ERT-poly(I:C), n = 6). Mice were then lethally irradiated and transplanted with LV.IDUA-transduced autologous BM-derived HSCs (10^6 cells/mouse). (A) At 12 weeks after transplantation, vector copy number was quantified by ddPCR in BM cells (VCN BM) of treated mice. Each dot represents the absolute VCN of a single mouse normalized on the mean of VCN of the ERT group; error bars indicate mean ± SEM. *p ≤ 0.05, Mann-Whitney test. (B) At 12 weeks after transplantation, the number of IDUA-specific CD8⁺ T cells was quantified by ELISPOT assay as SFU/10^5 CD8⁺ T cells isolated from the spleen of treated mice. Each dot represents one mouse; error bars indicate mean ± SEM. (C) Sera from ERT-poly(I:C)-treated mice were collected before (PRE) and 12 weeks after gene therapy (POST), and IDUA enzymatic activity was quantified fluorometrically. Dashed line indicates the threshold of positivity obtained as mean + 2SD of IDUA enzymatic activity in the sera of n = 17 untreated MPS-I mice (2.41 pmol/1 h/1 µL). IDUA enzymatic activity of each single mouse is shown.

we performed *ex vivo* HSC GT in ERT-poly(I:C) mice. Results showed that engraftment of IDUA-transduced HSCs was impaired in 4 of 6 ERT-poly(I:C)-treated mice (Figure 7A), and a high number of anti-IDUA CD8⁺ T cells was detected in the spleen of those 4 ERT-poly(I:C)-treated mice in which IDUA-transduced HSCs were completely eliminated (Figure 7B). On the other hand, detectable levels of IDUA enzymatic activity were quantified in sera of the 2 mice from the ERT-poly(I:C) group in which transduced HSCs engrafted (Figure 7C).

These findings demonstrate that anti-IDUA T cell responses could be triggered in MPS-I mice under ERT treatment in the presence of a pro-inflammatory stimulus.

DISCUSSION

Here we demonstrate the effect of pre-existing anti-transgene T cells on *ex vivo* HSC GT in the murine model of MPS-I. Specifically, we show that subcutaneous injection of IDUA in adjuvant promotes a pre-existing anti-IDUA immune response in MPS-I mice, which prevents the engraftment of LV.IDUA-transduced HSCs. While the presence of anti-IDUA IgGs does not impact on *ex vivo* HSC GT, IDUA-specific CD8⁺ T cells actively eliminate transplanted IDUA-overexpressing HSCs. Variable levels of transduced HSC engraftment can be achieved by treating pre-immunized MPS-I mice with T cell-depleting agents, i.e., anti-CD3 mAb or Fludarabine. Finally, we show that, in MPS-I mice, the activation of an innate response in concomitance with ERT can induce anti-IDUA CD8⁺ T cells, which affect the outcome of *ex vivo* HSC GT.

Pathological settings resulting from enzyme deficiencies can benefit from ERT. However, the development of unwanted immune responses against recombinant proteins represents a common issue.[20,21] The presence or absence of cross-reactive immunological material (CRIM) influences the severity of ERT-induced immunity, being more severe in CRIM-negative subjects harboring null mutations.[22,23] Accordingly, more than 90% of patients affected by the most severe form of MPS-I, the Hurler disease, develop anti-IDUA antibodies as a consequence of ERT.[18] The immunogenicity of IDUA, confirmed by the detection of anti-IDUA IgGs in the sera of MPS-IH patients, was not reproduced in MPS-I mice. Although MPS-I mice are IDUA knockout and, thus, CRIM negative, i.v. injection of rhIDUA, applied at the same dose and schedule used in patients, promoted anti-IDUA IgGs only in half of the treated animals. Moreover, IDUA-specific IgG concentration in sera was 1,000-fold lower compared to that observed in human samples. The poor and inconsistent anti-IDUA humoral response described in MPS-I mice was previously demonstrated, and it was independent of the amount of injected enzyme: high doses of enzyme also failed in promoting a homogeneous immunity in treated mice.[24] Moreover, the percentage of Ab-positive mice was shown to be higher when ERT was applied in adult compared to newborn MPS-I mice.[25] However, the *ex vivo* HSC GT required the treatment of mice at 4 weeks of age, thus limiting the use of ERT as a model for dissecting the role of pre-existing anti-IDUA immunity on GT.

To maximize the pre-existing anti-IDUA immunity, rhIDUA was subcutaneously injected with IFA into MPS-I mice. This procedure is known to favor antigen uptake by antigen-presenting cells (APCs), to mediate both presentation in the context of major histocompatibility complex (MHC) class II and cross-presentation in the context of MHC class I, and to promote efficient Ab production.[26] Anti-IDUA IgGs were homogenously induced at high levels, thus resembling the anti-IDUA humoral response observed in ERT-treated MPS-IH patients, and their presence did not impact on the efficacy of transduced HSC engraftment. The negligible impact of anti-transgene antibodies on *ex vivo* GT has been already demonstrated in GT for hemophilia A in FVIII-deficient mice, in which pre-existing anti-FVIII antibodies did not impair the efficacy of the treatment.[27] Conversely, we showed that IDUA-transduced HSCs are rejected in IFA-IDUA pre-immunized MPS-I mice as a result of a CD8⁺ T cell response that is specific to the transgene product. This mechanism recapitulates, to some extent, previous observations in the *in vivo* GT field, where cytotoxic CD8⁺ T cells specific for the transgene are *de novo* induced as a consequence of viral vector injection and severely hinder the efficacy of the treatment.[28–30]

In contrast to these data, we demonstrated that IDUA-overexpressing cells become the target of pre-existing transgene-specific $CD8^+$ T cells spared by irradiation. This result is in line with previous observations demonstrating that memory T cells are radio-resistant and maintain immunological memory.[31,32] Therefore, it can be speculated that residual IDUA-specific $CD8^+$ T cells in pre-immunized MPS-I hosts are re-activated and expanded upon exposure to IDUA-transduced cells and mediate the rejection of gene-corrected HSCs. As discussed above, in our model, IDUA-specific $CD8^+$ T cells might be generated via rhIDUA cross-presentation by APCs. However, we cannot exclude that the formation of immune complexes mediates the internalization of exogenous IDUA and favors its cross-presentation to $CD8^+$ T cells and the consequent priming.[33]

Thus far, only an anti-IDUA IgG response has been detected and characterized in MPS-I patients after ERT. It is known that anti-IDUA IgGs can alter the uptake and bio-distribution of the drug, but they have no clear effect on clinical efficacy.[24,34] Although the presence of IDUA-specific T cells in ERT-treated MPS-I patients has not been demonstrated yet, the interaction of B cells with antigen-specific T cells is typically required to drive Ab switching to an IgG isotype.[35,36] Moreover, both $CD4^+$ and $CD8^+$ T cells specific for acid alpha-glucosidase, a defective enzyme in Pompe disease, have been detected in patients after ERT.[37] Given these evidences, we cannot exclude the induction of anti-IDUA T cells in MPS-IH patients receiving ERT. Therefore, a deeper investigation of the ERT-induced T cell immunity in patients is warranted in order to better define its impact on protein or gene replacement therapies.

Autologous HSCT typically does not require the strong immunosuppressive protocols applied in allogeneic settings to avoid immune-mediated complications.[38] The myeloablative compound Busulfan was sufficient as conditioning regimen for MLD patients to eliminate the host's HSCs and favor engraftment of gene-corrected HSCs in previous *ex vivo* GT trials.[10,11] Nonetheless, the critical effect of pre-existing transgene-specific T cells that we demonstrated entails a reassessment of the conditioning regimen to include a lympho-ablative drug in other settings, such as in *ex vivo* HSC GT in immunocompetent subjects previously exposed to the transgene-encoded protein. In allogeneic transplantation settings, non-mitogenic anti-CD3 mAb can be employed to modulate T cells and to improve allogeneic transplantation outcome.[39–41] Moreover, anti-CD3-depleting Ab was previously demonstrated to prevent and modulate ERT-induced anti-acid-alpha-glucosidase immunity in the murine model of Pompe disease.[42]

In our hands, the use of anti-CD3 depleting Ab induced a profound T cell depletion in pre-immunized MPS-I mice, and it allowed engraftment of transduced HSCs. As an alternative, Fludarabine can be employed to modulate T cells: we showed that the association of lethal irradiation with Fludarabine improves the engraftment efficiency of gene-corrected HSCs in pre-immunized MPS-I mice, even if the lympho-depletion provided was suboptimal. This could be explained by the Fludarabine pharmacokinetic, which is different between mice and humans, making the maximum tolerated dose of Fludarabine 10 to 30 times lower in humans than in mice.[43,44] Fludarabine is currently applied in allogeneic HSCT for MPS-IH, and it is preferred over cyclophosphamide because of its reduced toxicity in association with Busulfan.[45,46] Accordingly, it has been chosen as a lympho-depleting agent in the preparative regimen of the phase I/II *ex vivo* GT clinical trial enrolling MPS-IH patients at San Raffaele Scientific Institute (ClinicalTrials.gov: NCT03488394).

A limitation of this study is the artificial immunization protocol used to overcome the poor immunizing effect exerted by ERT in MPS-I mice. Although this artificial immunization does not completely recapitulate the physiological immune response induced by ERT in MPS-I patients, it was required to dissect the role of humoral and cellular responses on *ex vivo* GT in MPS-I mice. The modest ERT immunogenicity in MPS-I mice can be explained by the absence of concomitant danger signals, such as tissue-damage mediators or activation via TLR agonists, during ERT administration in MPS-I mice, which is required to trigger innate immunity and license the priming of the adaptive immune system toward exogenous antigens.[47–51] The i.v. infusion of soluble antigens is indeed known to be poorly immunogenic in mice, since it provides little or no tissue damage, and antigens are rapidly cleared from the circulation, thus hindering the effective uptake from APCs.[52,53] Conversely, i.m. injection of soluble rhIDUA induced strong IDUA-specific humoral and cellular responses, possibly because of the danger signal provided after the disruption of the muscle tissue.

Innate immunity activation can also result from the concomitant exposure to pathogens and external stimuli.[50] We showed that mimicking a concomitant viral infection by providing the TLR3 agonist poly(I:C)[54] during ERT promotes a homogeneous anti-IDUA IgG response and induces anti-IDUA $CD8^+$ T cells in MPS-I mice, which in turn prevented IDUA-transduced HSC engraftment. Thus, both i.m. injection of IDUA and treatment with poly(I:C) can improve ERT immunogenicity, but the intensities of humoral and cellular responses induced varies and do not correlate. Different immunization protocols can indeed promote different responses, either skewed toward the anti-IDUA T cell or Ab response. It cannot be excluded that the strong anti-IDUA humoral response we and others[55–58] observed in MPS-IH patients after ERT may be due to the adjuvant effect exerted by concomitant exposure to environmental stimuli. Therefore, we believe that the use of a stronger immunization protocol to investigate the effects of pre-existing anti-IDUA immunity on *ex vivo* HSC GT was justified by the immunological discrepancies observed between mice and humans.

Overall, our findings demonstrate the importance of monitoring and controlling pre-existing immunity against protein replacement therapy in MPS-IH patients before and after *ex vivo* HSC GT. The rejection mechanism here described can take place in other pathological settings where similar immunological premises are occurring, and it indicates that the same caution should be applied in GT protocols involving immunocompetent patients pre-exposed to

and immunized against the transgene-encoded proteins. Accordingly, the inclusion of the lympho-depleting drug Fludarabine as preparative conditioning protocol before *ex vivo* GT in the context of the phase I/II clinical trial for MPS-IH (ClinicalTrials.gov: NCT03488394) ensures the modulation of possible pre-existing anti-IDUA immunity to favor the engraftment and the therapeutic efficacy of IDUA-corrected HSCs.

MATERIALS AND METHODS

Mice

The *idua*$^{-/-}$ mice (C57BL/6 background)[59] were imported in our animal facility as a kind gift of professor J.M. Heard and crossed with C57BL/6 mice from Charles River. Progeny was intercrossed to obtain an inbred strain. Mice were genotyped to identify *idua*$^{-/-}$ mice (MPS-I), and *idua*$^{+/-}$ and i*dua*$^{+/+}$ siblings were used as BM donors. C57BL/6-Ly5.1 mice were purchased from Charles River. Experimental mice were randomized according to the gender.

Anti-IDUA IgG Concentration in Human Samples

Peripheral blood was obtained from MPS-IH patients and healthy subjects in accordance with local committee approval (TIGET05 and TIGET09) and with the Declaration of Helsinki. Plasma was collected after centrifugation of EDTA blood samples, and anti-IDUA IgG concentration was determined by ELISA. Briefly, 0.2 μg rhIDUA (Aldurazyme, Genzyme) was coated onto the wells of a 96-well microtiter plate (Thermo Fisher Scientific). After 12–16 h of incubation at 4°C, the plate was blocked for 2 h at room temperature (RT) with PBS-5% BSA (Roche), and diluted plasma samples were plated. After an additional 2 h at RT, biotinylated anti-human immunoglobulin (Thermo Fisher Scientific) was added to the plate for 2 h at RT, followed by 45-min incubation with Streptavidin horseradish peroxidase (HRP) conjugate. A substrate solution containing o-Phenylenediamine dihydrochloride (OPD) was prepared according to the manufacturer's instructions (Sigma-Aldrich, P4664), and the colorimetric reaction was stopped after 6–10 min by the addition of sulfuric acid (stop solution, R&D Systems). Absorbance was read at 492-nm wavelength using SkaltRE for Multiskan go version 3.2 (Thermo Scientific). IgG concentration was determined by comparison to a serial dilution of a known-concentration solution of human IgGs (Sigma-Aldrich), run in parallel.

ERT and Immunization Protocols

rhIDUA (Aldurazyme, Genzyme) was diluted in PBS and weekly injected i.v. in 4-week-old MPS-I mice. Mice received 5 injections. Doses were adjusted according to body weight (0.58 μg/g).

PBS, rhIDUA, or OVA protein was emulsified with IFA (Sigma-Aldrich) and subcutaneously injected nearby the inguinal lymph nodes of 4-week-old MPS-I mice. 50 μL emulsion was administered to each side (total 29 μg protein/mouse).

To mimic viral infection, 4-week-old MPS-I mice received ERT as described above. Second and third doses were administered together with 50 μg/mouse poly(I:C) (tlrl-pic, InvivoGen).

Anti-IDUA or Anti-OVA IgG Quantification and Isolation

IDUA or OVA-specific IgGs were quantified in the sera of mice by ELISA. rhIDUA or OVA (Sigma-Aldrich) was used to coat microtiter plates (0.2 μg/well in 0.1 M carbonate buffer [pH 9.6]). After 12–16 h of incubation at 4°C, the plate was blocked for 2 h at RT with PBS-1% BSA (Roche). Diluted sera were added, and, after an additional 2 h at RT, anti-IDUA or anti-OVA antibodies were detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Sigma-Aldrich). Plates were reacted with H_2O_2 and OPD and read at 492-nm wavelength using SkaltRE for Multiskan go version 3.2 (Thermo Scientific). IgG concentration was determined by comparison to a serial dilution of a known-concentration solution of murine IgGs (554721, BD Biosciences), run in parallel.

Sera from IDUA-IMM and OVA-IMM MPS-I mice were collected and used for IgG purification. Pre-packed spin columns from GE Healthcare (Ab Spin Trap, 28-4083-47) were used according to the manufacturer's instructions. Purified IgGs were quantified by ELISA as described above and i.v. injected into naive mice.

Cytokine Production

Spleens from experimental mice were collected and processed into a single cell suspension. 0.7×10^6 splenocytes were plated in triplicate in 96-well plates in the presence of 10 μg/mL rhIDUA in complete RPMI 1640 (Lonza, Switzerland) with 10% fetal bovine serum (FBS) (Euroclone, ECS0180L), 100 U/ml penicillin/streptomycin (Lonza, 17-602E), 2 mM L-glutamine (Lonza, 17-605E), Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA) (Gibco, 11140-035), 1 mM Sodium Pyruvate (Gibco, 11360-039), 50 nM 2-Mercaptoethanol (Gibco, 31350-010). After 96 h of culture, supernatants were collected and tested for cytokine production with Bio-Plex Pro Mouse Cytokine Th1/Th2 assay (Bio-Rad), according to the manufacturer's instructions.

ELISPOT Assays

$CD8^+$ T cells were magnetically isolated from the spleen of experimental mice (Miltenyi Biotec, 130-104-075). 10^5 $CD8^+$ T cells were plated in triplicate in ELISPOT plates (Millipore, Bedford, MA) pre-coated with anti-IFN-γ capture mAb (2.5 μg/mL; BD Pharmingen, R46A2) in the presence of IL-2 (50 U/mL; BD Pharmingen) and 10^5 irradiated (6,000 rad) untransduced or LV.IDUA-transduced autologous EL-4 cells. After 42 h of incubation at 37°C and 5% CO_2, plates were washed and IFN-γ-producing cells were detected by biotin-conjugated anti-IFN-γ mAb (0.5 μg/mL; BD Pharmingen, XMG 1.2). Streptavidin-HRP conjugate (Roche) was added. Total splenocytes or total BM (0.35×10^6 cells/well) was plated in complete RPMI in triplicate in ELISPOT plates pre-coated with rhIDUA (2 μg/well). After 24 h of incubation at 37°C and 5% CO_2, plates were washed and anti-IDUA IgG-secreting cells were detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Sigma A2554). All plates were reacted with H_2O_2 and 3-Amino-9-ethylcarbazole (Sigma, A6926). Spots were counted by ImmunoSpot reader (Cellular Technology).

Ex Vivo HSC GT

BM from 6-week-old *idua*$^{+/-}$ and *idua*$^{+/+}$ mice was harvested by flushing femurs. HSCs were purified for lineage^{-} selection using the Lineage Cell Depletion Kit (Miltenyi Biotec, 130-090-858), according to the manufacturer's instructions, and they were transduced at an MOI of 100 with IDUA- or OVA-encoding LV (LV.IDUA, LV.OVA). All LVs were produced by transient four-plasmid transfection of HEK293T cells, as previously reported.[60]

MPS-I mice received total body irradiation (935 cGy split in two doses performed at least 2 h apart) and were i.v. injected with LV-transduced cells (10^6 cells/mouse) on day 0. IDUA-IMM mice received irradiation and transplantation 4 weeks after immunization. At weekly intervals, mice were bled and the engraftment of donor cells was determined by flow cytometry. In some experiments, IDUA-IMM MPS-I mice were i.v. injected with depleting anti-CD8 Ab (clone YTS169.4, BioXcell, 250 μg/mouse) 1 day before transplantation of transduced cells. After transplantation, mice were weekly i.v. injected with anti-CD8 Ab (clone 53-6.7, BioXcell, 250 μg/mouse). In some experiments, IDUA-IMM MPS-1 mice were i.v. injected with anti-CD3 depleting Ab (clone 145-2C11, BioXcell, 100 μg/mouse) 4 days before transplantation of transduced cells. After transplantation, mice were weekly i.v. injected with anti-CD3 Ab (25 μg/mouse) for 7 weeks. In some experiments IDUA-IMM MPS-I mice were intraperitoneally injected with a total amount of 1,000 μg/g Fludarabine Phosphate (Accord); the total amount of drug was divided into 3 administrations performed on days −4, −3, and −2 before transplantation.

Engraftment Quantification

VCN was measured on BM and peripheral blood cells to quantify engraftment of transduced HSCs. Genomic DNA was isolated with QIAamp DNA Blood Mini Kit (QIAGEN, 51106), according to the manufacturer's instructions. Vector integrations were quantified by QX200 Droplet Digital PCR System (Bio-Rad), according to the manufacturer's instructions. Primers and probes were designed on the vector sequence and on control sequence used for normalization (mouse *Sema3a* gene).

Flow Cytometry

Peripheral blood was harvested by temporal vein puncture and mixed with NaCitrate (5:1 ratio). 30 μL blood was incubated with 25 μL of a calibrated suspension of fluorescent beads (CountBright Absolute Counting Beads, Thermo Fisher Scientific, C36950) and stained with the following Abs: purified anti-CD16/CD32 (2.4G2), fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (17A2), BD Horizon Brilliant Violet 786 (BV786)-conjugated anti-CD4 (RM4-5), Pacific Blue-conjugated anti-CD8 (53-6.7), R-phycoerythrin (PE)-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated anti-CD19 (1D3), and allophycocyanin-Cy7-conjugated anti-B220 (RA3-62B) (all from BD Biosciences) for 30 min at 4°C. Red blood cells lysis was performed with BD Phosflow (BD Biosciences, 558049), according to the manufacturer's instructions. Labeled cells were washed two times with PBS 1% FBS and acquired with a BD LSRFortessa analyzer, and results were analyzed with FlowJo 10 software. T cell populations were identified after exclusion of the CD11b^{+} CD19^{+} and B220^{+} cells. The absolute number of cells per microliter was obtained as the ratio of beads events to cells events.

IDUA Enzymatic Activity

Enzyme activity was measured fluorometrically[61] following the incubation of 10 μL serum for 1 h at 37°C with 10 μL substrate (2 mM 4-methylumbelliferyl α-L-iduronide, Glycosynth) in 0.1 M Na formate buffer (pH 3.2). Fluorescence of the 4-methylumbelliferone released was measured after the addition of 1 mL 0.5 M carbonate buffer (pH 10.7). Fluorescence was read at 365 nm (excitation) and 448 nm (emission) on a PerkinElmer fluorometer.

Statistics

All statistical analysis was performed using non-parametric Mann-Whitney U test (two-tailed); p values below 0.05 were considered significant.

Study Approval

All the mice were maintained in specific-pathogen-free (SPF) conditions, and the procedures involving animals were designed and performed with the approval of the Animal Care and Use Committee of the San Raffaele Hospital (IACUC #830) and communicated to the Ministry of Health and local authorities according to Italian law. Peripheral blood was obtained from MPS-IH patients and healthy subjects upon informed consent signature in accordance with the Ethical Committee of San Raffaele Scientific Institute (protocols TIGET05 and TIGET 09).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.04.014.

AUTHOR CONTRIBUTIONS

G.S. designed and conducted the experiments, analyzed and interpreted data, and wrote the manuscript. L.P. conducted and analyzed experiments with patient samples and revised the manuscript. F.F. provided reagents and protocols. C.L. and D.T. provided help in some experiments. F.D., M.A.D., and S. Gasperini provided patient samples and critically discussed the data. B.G. provided the LV.IDUA-encoding vector and scientifically revised the manuscript. M.E.B., A. Aiuti, and L.N. scientifically revised the manuscript. A. Annoni supervised experimental design and data interpretation and helped in manuscript writing. A.B. conceived the scientific idea, helped in data interpretation, and scientifically revised the manuscript. S. Gregori conceived the scientific idea, supervised experimental design and data interpretation, and wrote the manuscript. All authors contributed to and approved the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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